MASTER THESIS IN CANCER BIOLOGY

Mechanistic investigation of *Myo*-Inositoltrispyrophosphate as a hypoxia modulator in combination with radiotherapy

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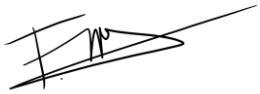
Statement of Authorship

I declare that I have used no other sources and aids than those indicated. All passages quoted from publications or paraphrased from these sources are indicated as such, i.e. cited and/or attributed. This thesis was not submitted in any other form for another degree or diploma at any university or other institution of tertiary education.



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Summary

Cancer is a leading cause of death worldwide. Despite several treatment options such as radiotherapy, surgery, chemotherapy and molecularly targeted therapy, and combinations of those, the fight against cancer is not satisfying yet. There is no “miraculous cure” and there will never be, since “cancer” is only a broad designation of a malady that covers a huge amount of different forms and behaviors. The only thing in common is the degenerated control of cells that start to grow uncontrollable, achieved through many different mechanisms. Given that, the work on the improvement of existing methods to counteract cancer, and on finding and developing new strategies, is of great importance.

Radiotherapy is one of the most commonly used treatment modalities, alone or in combination with another. It induces a complex network of secreted factors that can stimulate tumor outgrowth, dissemination, incomplete tumor regression and immune reactions. Irradiation can contribute to tumor eradication by damaging cells, respectively their DNA, in an amount that cannot be repaired anymore and will lead to mitotic catastrophe that either ends in senescence or death of the cell. DNA damage by irradiation is achieved through the formation of reactive oxygen species (ROS), generated through radiolysis. ROS are highly reactive and will lead to damage of the DNA and other macromolecules.

The response and treatment outcome of irradiation is dependent on several factors, described as the 5 Rs of radiotherapy. These include the repair capacity of the cells and their intrinsic radiosensitivity, depending on their position within the cell cycle. After irradiation, the cells will redistribute within the cell cycle and repopulate in order to fill up a void created by irradiation-induced cell death. The fifth R addresses the reoxygenation between two dose fractions. Reoxygenation is crucial because oxygen plays a key role in radiotherapy. It is necessary for the stabilization (“fixation”) of damage induced by ROS. Without sufficient oxygen, the survival fraction of irradiated cells is 2.5 to 3 times higher as compared to normal oxygenated cells. For this reason, the oxygen status of the tumor is a major predictor and influencer of radiotherapy treatment outcome. There are several strategies available to increase the amount of oxygen in the blood, and therefore its delivery to a tumor, but they are still not fully gratifying.

In this Master’s Thesis, a novel compound, *Myo*-Inositoltrispyrophosphate (ITPP), was investigated. It was developed as an effector of hemoglobin to lower the affinity of hemoglobin to oxygen. Thereby an enhanced release of oxygen e.g. in hypoxic tumors can be achieved. So far, the mode of action remains unclear. Further, ITPP has structural similarities to PIP_2 and PIP_3, which are second messengers upstream of the Phosphoinositide 3-kinase / Protein kinase B (PI3K/AKT) pathway, which can influence tumor growth and progression.

This Master's Thesis demonstrates that ITPP can modulate the availability of oxygen within a tumor and reoxygenate hypoxic tissue. Through the increase of oxygen, the radiosensitivity increases, leading to a significant growth delay *in vivo,* when radiotherapy is combined with ITPP. This was demonstrated in an efficacy-oriented experiment of A549 xenografts and in FaDu xenografts. As part of a broad mechanistic investigation, histological analysis of tumor oxygenation and of DNA double strand breaks (DSB) by γH2AX staining was performed. A significant increase in DNA DSBs in initially hypoxic tumor zones could be achieved with ITPP in combination with radiotherapy. Through irreparable damage like DNA DSBs, cells will experience radiation-induced loss of clonogenicity, primarily via mitotic catastrophe or other modes of cell death.

These results suggest that a tumor growth delay achieved through a combined treatment modality of ITPP and irradiation underlies the induction and "fixation" of DNA DSBs, not only in normoxic but also in reoxygenated tumor zones due to ITPPs oxygen-modulatory effect.

Furthermore, ITPP has also an impact on the PI3K/AKT pathway *in vitro,* as it upregulates PTEN levels and decreases pAKT concomitantly.

In summary, this Master's Thesis presents mechanistic investigations on the treatment modality of ITPP in combination with radiotherapy *in vivo* through the oxygen-modulating capabilities of ITPP: more ROS can be produced through irradiation, leading to the *oxygen-enhancing effect* within hypoxic areas of the tumor and more extended induction of DNA-double strand breaks. Therefore, ITPP is claimed as a potent radiosensitizer and neoadjuvant treatment option for hypoxic tumors.

Zusammenfassung

Krebs ist eine der häufigsten Todesursachen weltweit. Trotz verschiedenen Behandlungsmöglichkeiten wie Radiotherapie, Chemotherapie, «molecularly targeted» Therapie oder die chirurgische Entfernung von Krebsgewebe, sowie Kombinationen derjenigen, ist die Behandlung von Krebs noch nicht zufriedenstellend. Eine Art «Wundermittel» wurde bislang nicht gefunden und wird es auch nicht geben, da Krebs nur ein Überbegriff für ein breites Spektrum von deregulierten Zellformen und -verhalten ist. Die einzige Gemeinsamkeit stellt das unkontrollierbare Wachstum und die stetige Veränderung der vielen Krebsformen dar. Deshalb ist es von grosser Wichtigkeit, die bestehenden Behandlungsmöglichkeiten zu verbessern und stetig neue Strategien zu finden und zu entwickeln.

Radiotherapie ist eine der häufigsten Behandlungsmodalitäten, alleine oder als Kombination. Durch Bestrahlung werden verschiedene Antworten provoziert, die von komplexen Signalnetzwerken über Immunreaktionen bis zur Umprogrammierung der Krebszellen geht. Zur Vernichtung von Krebszellen trägt Bestrahlung durch die Bildung von reaktiven Sauerstoff-Spezies (ROS) bei, welche hoch reaktiv sind und DNA und andere Makromoleküle schädigen können. Wenn DNA Doppelstrangbrüche (DSB) nicht repariert werden, kann sich eine Zelle nicht korrekt teilen und es kommt zur mitotischen Katastrophe.

Die Reaktion des Krebsgewebes und der Behandlungserfolg der Bestrahlung hängt von verschiedenen Faktoren ab, die man gemeinhin als die 5 Rs der Radiotherapie bezeichnet. Dazu gehört die Reparaturkapazität der Zellen, DNA DSB effizient zu reparieren (repair). Des Weiteren spielt die Position im Zellzyklus eine wichtige Rolle – je nach dem in welcher Phase sich eine Zelle befindet, ist sie mehr oder weniger bestrahlungssensitiv (radiosensitivity). Ausserdem kommt es zu einer Umverteilung der Zellen innerhalb des Zellzyklus (redistribution) nach einer Bestrahlung, da einige Zellen in ihrem Zyklus weiterfahren können, wohingegen andere blockiert sind (durch DNA Schäden). Nach einer Bestrahlungsdosis werden einige Zellen irreparable Schäden aufweisen. Es wird ein Programm gestartet, welches die Entstehung von Zellklonen mit unvollständigen oder falsch organisierten Chromosomensätzen verhindern soll. Das Programm führt entweder zum Tod oder zur Seneszenz der Zelle. Die verbleibenden Krebszellen werden versuchen, die entstandene Lücke aufzufüllen (repopulation). Das letzte R adressiert die Reoxygenierung zwischen zwei Bestrahlungsfraktionen (reoxygenation). Reoxygenierung ist ein wichtiges Thema, da der Behandlungserfolg weitgehend vom verfügbaren Sauerstoff im Tumor abhängt. In einem schlecht oxygenierten Tumor ist die Reaktion, beziehungsweise die Summe der induzierten und stabilisierten DNA DSB, 2.5 bis 3 Mal geringer im Vergleich zu einem normal oxygenierten Tumor. Durch Bestrahlung kommt es zur Radiolyse und zur Bildung von ROS. Für die Stabilisierung des ROS-induzierten Zellschadens ist Sauerstoff essentiell und zählt zu einem der wichtigsten Indikatoren und

Faktoren des Behandlungserfolges. Es gibt schon verschiedene Lösungsansätze für hypoxische Tumoren um den Sauerstoffgehalt zu erhöhen, doch noch keine, welche vollumfassend wirkt.

Myo-Inositoltrispyrophosphate (ITPP) ist ein vielversprechendes Molekül, welches die Sauerstoffaffinität von Hemoglobin manipulieren kann. Des Weiteren hat es Ähnlichkeit zu den beiden sekundären Botenstoffen PIP_2 und PIP_3, welche oberhalb des Phosphatidylinositol-3-Kinase / Protein Kinase B (PI3K/AKT)-Signalweges zu finden sind. Der PI3K/AKT Signalweg kann die Tumorprogression und das -wachstum fördern.

Diese Masterarbeit zeigt, dass ITPP durch seine Hemoglobin-modulierenden Eigenschaften den Sauerstoffgehalt in Bereichen mit geringem partiellen Sauerstoffdruck erhöhen kann. Da der Sauerstoff den Behandlungserfolg durch Bestrahlung massgeblich erhöht, kann ITPP als Radiosensitizer *in vivo* eingeordnet werden. Dies zeigt sich in einer signifikanten Tumor-Wachstumsverzögerung. In einer breit angelegten mechanistischen Untersuchung wurde eine Tumor-Histologie für den DNA DSB Marker γH2AX durchgeführt. Durch ITPP in Kombination mit Bestrahlung konnten signifikant mehr DNA DSB in initial hypoxischen Regionen der Tumorspezies gezählt werden, verglichen mit einer Kontrollbestrahlung. So konnte gezeigt werden, dass der Effekt von Bestrahlung plus ITPP der Induktion und Stabilisierung von DNA DSBs in hypoxischen Zonen zugrunde liegt, die sonst bestrahlungsinsensitiv sind.

Ausserdem zeigt ITPP auch einen Effekt auf den PI3K/AKT Signalweg *in vitro*, da es durch ITPP zu einem erhöhten Level von PTEN kommt und zu einer Verminderung von pAKT.

Zusammengefasst präsentiert diese Masterarbeit mechanistische Hinweise für die Verzögerung des Tumorwachstums durch ITPP in Kombination mit Bestrahlung *in vivo* durch den Sauerstoff-modulierenden Effekt von ITPP: ROS werden durch Bestrahlung gebildet und schädigen die DNA. DNA Schäden wie DSB können durch den nun vorhandenen Sauerstoff fixiert werden. Nicht reparierte DNA DSB führen zur mitotischen Katastrophe der entsprechenden Krebszellen. Deshalb kann man ITPP als Radiosensitizer einordnen und zur Kombination mit Bestrahlungstherapie für hypoxische Tumoren empfehlen.

Graphical Abstract

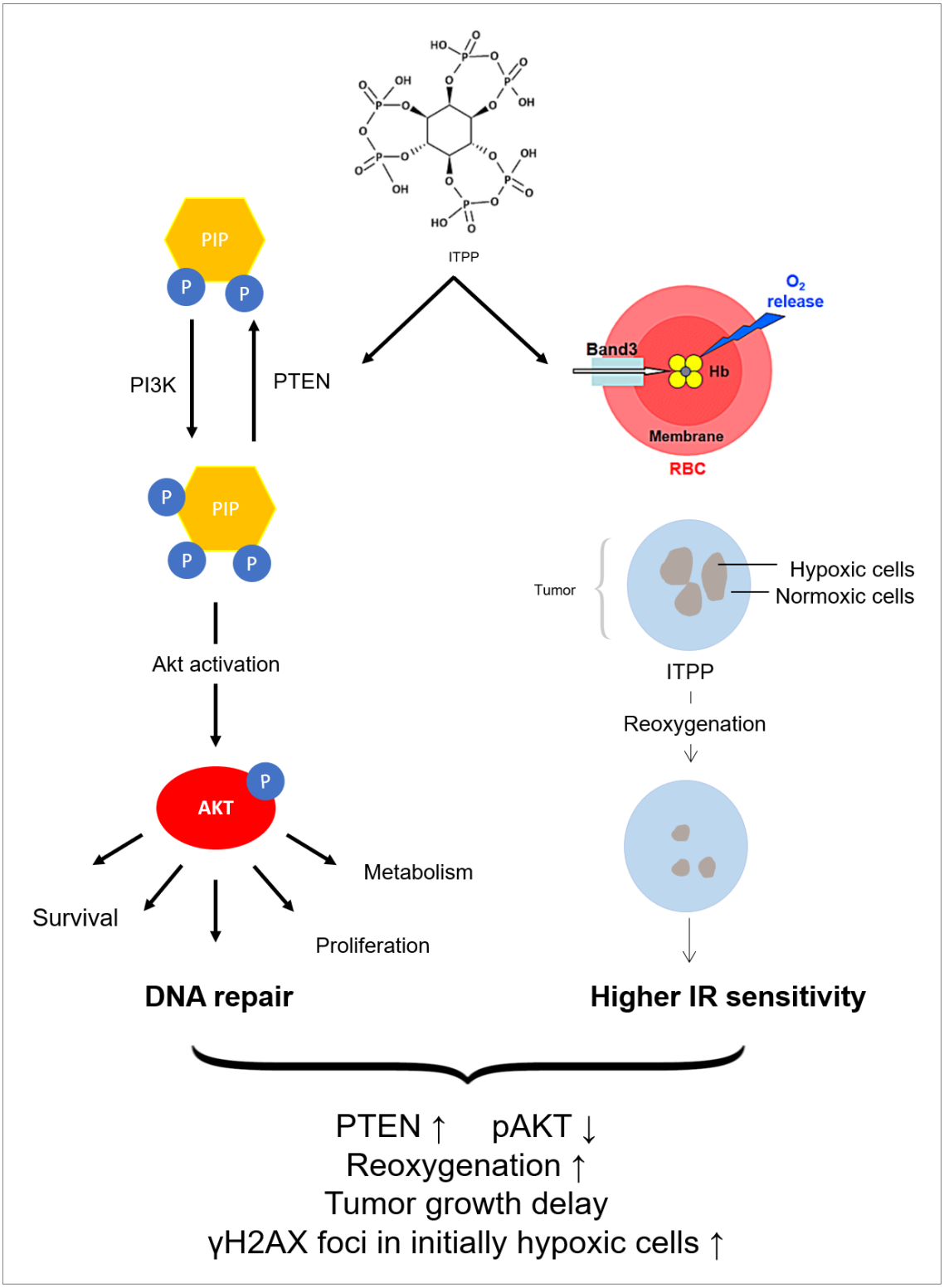

Figure 1 Overview of the dual function of ITPP. ITPP modulates the affinity of hemoglobin to oxygen and interferes with the PI3K/AKT pathway. Thereby, ITPP might reoxygenate hypoxic tissues, which can be exploited for radiotherapy. Figures adapted from [40,42,47].

Abbreviations

2,3 BPG	2,3 Bisphosphoglyceric acid
AKT	see PKB
CA-IX	Carbonic anhydrase 9
DDR	DNA damage response
DNA-PK	DNA protein kinase
DSB	Double strand break
H & E	Hematoxylin and eosin
HIF	Hypoxia inducible factor
HNSCC	Head and neck squamous cell carcinoma
HR	Homologous recombination
H2AX	Variant of histone 2A
γH2AX	phosphorylated H2AX
GLUT1	Glucose transporter 1
Gy	Gray
ITPP	*Myo*- Inositoltrispyrophosphate
mAb	monoclonal Antibody
NSCLC	Non-small cell lung carcinoma
NHEJ	Non-homologous end-joining
PI3K	Phosphatidylinositol-3-kinases
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein kinase B
PTEN	Phosphate and tensin homolog
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
VEGF	Vascular endothelial growth factor

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1 Introduction

1.1 Characteristics of cancer

Cancer is a broad designation for an extended group of different malignancies. It includes all types of neoplastic growth, starting from a benign, but abnormal cell accumulation, resulting in the highly deadly and aggressive invasions or spread of cancer cells to other parts of the body. Cancer has not a single cause, it is a progressive multistep disorder that transforms normal cells into cancer cells. Several mutations in genes controlling cell proliferation and homeostasis must be acquired to transform a normal and healthy cell into a malignant, potentially deadly cell. Features that allow cells to become cancer cells include traits that enable them to survive, proliferate and disseminate. These different characteristics were first summarized by Robert Weinberg and Douglas Hanahan in 2000: "The Hallmarks of Cancer" concentrate the complexity of cancer into six main characteristics, that are required to become a malignant, invasive cancer cell. The key traits contain sustained proliferative signaling, insensitivity to growth suppression, evasion of cell death signaling, limitless replicative potential, activation of angiogenesis, invasion into surrounding tissues, and the formation of distal metastases. In contrast, normal and healthy cells ensure an appropriate cell number, proper tissue architecture and function by strictly controlling their growth and behavior.

The most characteristic trait of cancerous cells is their uncontrollable proliferation. They evade or escape growth suppression, or even apoptotic signaling and moreover, they produce growth factors themselves, resulting in a vicious circle of unstoppable growth. In healthy cells, several tumor suppressors are active to control proliferation or start a cell death program to avoid extended amounts of cells, or as a solution for cells with irreparable damage. Apoptosis serves as a natural barrier to develop cancer as it eradicates potential dangerous cells. Through sustaining the proliferative signaling and evading cell death, cancer cells have an unlimited proliferative capacity. In addition, replicative senescence often can be bypassed. For this fast-replicative behavior, cancer cells need to modify their energy metabolism towards glycolysis, and because of their large demand of nutrients, they must induce angiogenesis in the surrounding vasculature so that new blood supply can feed the needs of the tumor. After a primary excessive growth of cells building a so-called neoplasm, local invasion into the host tissue follows. Afterwards, an eventual invasion of tumor cells into the blood stream and dispersion all over the body occurs in a process called metastatic cascade. Another key trait of cancer is to evade the recognition of the immune system. Some cancer types are even infiltrated with immune cells that can cause a local inflammatory response, resulting in more growth and survival factors and enzymes

facilitating angiogenesis by modifying the surrounding extracellular matrix. Those additional traits were added to the initial six hallmarks, resulting in ten hallmarks of cancer [1, 2].

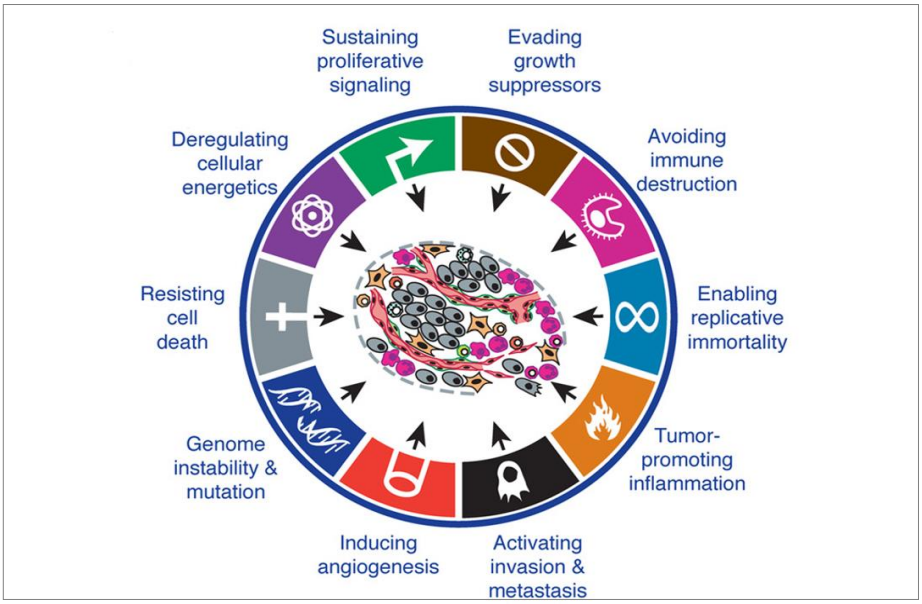


Figure 2 A graphical summary of the hallmarks of cancer. Figure adapted from [1].

1.2 Cancer Treatment Strategies

With increasing knowledge about mechanisms and characteristics of cancer, treatment has changed from relatively general cytotoxic agents to selective and mechanism-oriented therapeutics. The choice of the treatment used is based on different characteristics of the cancer addressed: the stage, location within the body, grade, genetic makeup, metastatic status and the general health status of the patient.

In general, cancer can be treated by surgery, radiotherapy, chemotherapy, molecularly targeted therapy and combinations of those. If the cancer is in an early stage and locally limited to one location, surgery is commonly and successfully used.

To eliminate potential danger of residing cells, radio- or chemotherapy can be used as part of a multimodality therapy. For insights into the stage of a tumor, surgical biopsies are necessary. This is important for prognosis and the decision for an appropriate treatment strategy. In severe cases, palliative care can be the last reasonable option.

1.2.1 Radiotherapy

Radiotherapy (also called X-ray therapy or irradiation) includes the usage of ionizing radiation composed of particles, that carry enough energy to liberate electrons from atoms or molecules. It can be administered by an external beam or internally via brachytherapy. Usually, external radiotherapy with ionizing radiation is generated and delivered by a linear accelerator.

The “absorbed dose” characterizes the amount of energy delivered to matter (especially living tissues) by irradiation and reflects the amount of energy deposited per mass. The International System of Units describes the absorbed dose as the “Gray” (Gy), which is equal to 1 joule/1 kilogram of matter. 1 Gy represents the amount of irradiation required to deposit 1 joule of energy in 1 kilogram [3].

Through irradiation, DNA can be damaged, leading to radiation-induced loss of clonogenicity. Since cancer cells are highly proliferative, they often are in a phase of the cell cycle where they are especially prone to DNA damage, in contrast to healthy cells, which are generally less proliferative. Through radiolysis, ROS are being produced, leading to several interferences in signaling pathways, repair processes and destruction of macromolecules like DNA (Figure 3) [4].

Radiotherapy can be curative in several different cancer types, if the tumor is localized in one area of the body. It can also be part of adjuvant therapy to prevent tumor recurrence after surgery to remove a primary malignant tumor (for example an early stage breast cancer). Further, irradiation acts synergistically with chemotherapy and can be used before, during and after chemotherapy in susceptible cancers. The susceptibility to irradiation is mostly determined by five factors, commonly known as the 5 Rs of radiotherapy [3, 5].

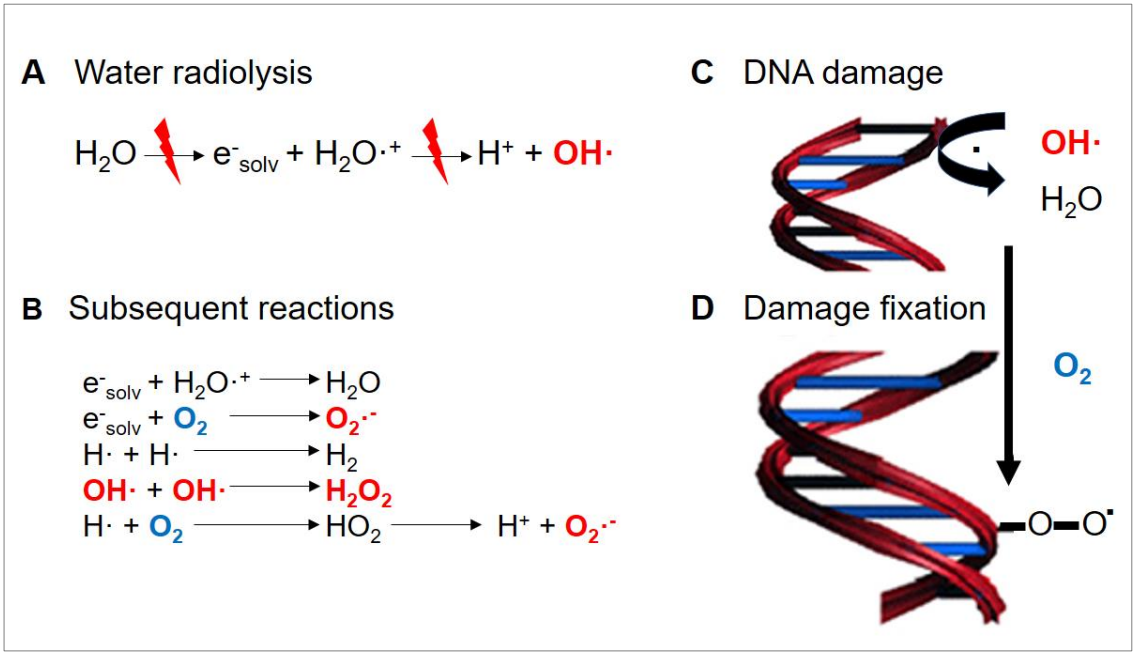


Figure 3 Irradiation induces the ionization and destabilization of water, leading to the formation of reactive radical species. **A)** These species react with neighboring molecules, forming ROS. **B)** The hydroxyl radical (OH•) is the most cytotoxic among the formed ROS. When generated in the proximity of DNA, they can attack DNA. **C)** This results in the formation of a DNA radical, which is easily reversible. But in the presence of oxygen, the DNA damage can be stabilized through oxidation of the DNA radicals. This leads to the formation of DNA peroxides. **D)** The oxygen dependent “fixation” of the DNA damage is known as the *“oxygen enhancing effect*”. Figure adapted from [4].

The 5 Rs of radiotherapy

The success to kill or minimize cancer cells by localized radiotherapy depends on several factors and the interactions of them. Initially, 4 Rs were described by Withers, which include the repair of sublethal DNA damage, cell repopulation, redistribution of cells within the cell cycle and the reoxygenation of hypoxic tumor areas [6]. An additional fifth R, intrinsic radiosensitivity, was later enclosed by Steel [7]. The optimization of these key biological parameters improves the local tumor control and minimizes the normal tissue toxicity. **Repair or recovery** involves the efficient repair of lesions such as DNA DSBs. Most of the radiation-induced DNA damage is sublethal and therefore it is effectively repaired in normal cells. However, cancer cells have abrogated DNA repair mechanisms. DNA DSBs that remain unrepaired can lead to mitotic catastrophe and other modes of cell death. The radiation sensitivity varies among the different phases of the cell cycle. In general, cells are most radiosensitive in M and late G2 phase and more resistant in late S phase. This pattern of radiosensitivity correlates with the mechanism of DNA DSBs repair. The DNA damage in the late S phase is repaired by homologous recombination (HR). HR is not as error prone as repair by non-homologous end joining (NHEJ), occurring in the early part of the cell cycle. As cancer cells are fast dividing, they are heterogeneously distributed in the cell cycle phases. By applying the first dose of irradiation, the most sensitive cells will experience mitotic catastrophe or another mode of cell death. The remaining cells eventually progress into the next cell cycle phase, which can be more radiosensitive compared to the previous one. This is called **redistribution**. Therefore, fractionation of a dose into two or more fractions is used to successfully eradicate most of the cancer cell population. The damage caused by irradiation can induce accelerated repopulation as the cells try to fill up the void created by the cells undergoing cell death. Because of this reason, **repopulation** is a common reason for the failure of conventional hypo-fractionated courses of radiotherapy. **Reoxygenation** of the cancerous tissue between two dose fractions improves the efficacy of radiotherapy and enhances tumor radiosensitivity. Additionally, tumor regions of acute or chronic hypoxia are associated with poor prognosis and aggressive tumor progression [3, 6-8].

1.2.2 Chemotherapy

Chemotherapy includes compounds that interfere with the cell's ability to replicate or to divide the chromosomes properly during mitosis. Compounds include **alkylating agents**, which are highly reactive and form bridges between DNA strands. Because of that, the replication machinery cannot proceed. **Antimetabolites** are compounds that can impede DNA or RNA synthesis by competing for the active site of an essential enzyme, or molecules that belong to any protein synthesis pathway. **Topoisomerase inhibitors** counteract with the function of the topoisomerase enzyme, which is essential for detangling DNA strands during replication. Another class of chemotherapeutic agents

presents **microtubule-interfering agents**, which either prevent the formation of microtubules, or their degradation. This has an impact on spindle formation during mitosis, in which doubled chromosomes are brought apart for cell division [9].

Of course, chemotherapeutic agents are not specific to cancer cells, leading to treatment-induced-toxicity in certain tissues, that have a high proliferation rate like skin and hair cells or cells of the intestinal linings. Chemotherapy is usually a combination of more than one cytotoxic agents and is generally also coupled with another treatment strategy.

1.2.3 Surgery

In general, tumors start to grow locally and then spread from the site of formation. In theory, non-hematological cancers can be cured if they are entirely removed by excision. The goal of surgery can be to remove the tumor alone or the whole organ affected. Examples of surgical procedures for cancer include mastectomy for breast cancer, prostatectomy for prostate cancer and lung cancer surgery for non-small cell lung cancer. Unfortunately, even a small tumor can already spread to other sites within the organism and form metastases. Also, a single cancer cell can regrow to another tumor, called recurrence. For this reason, a pathologist will examine each excised specimen to determine if a margin of healthy tissue is present and thus decreases the chance, that microscopic cancer cells are left in the patient. In addition to removal of the primary tumor, it is also necessary for staging. Surgery is often performed in combination with other treatments such as chemotherapy or radiotherapy. So far it is not clear if surgery before or after another adjuvant treatment has an impact on the survival rate [10, 11].

1.2.4 Molecularly targeted therapy

This class of treatment modality includes several compounds that block the growth of cancer cells by interfering with specific targeted molecules needed for tumorigenesis and tumor progression. The most successful targeted therapies include compounds that target a protein or enzyme that carries a mutation or other genetic alteration that is specific to a cancer entity and not found in the normal host tissue. The target specificity reduces side effects within the normal tissue as compared to chemo- or radiotherapy. Chemo- or radiotherapy only focuses on the eradication of fast proliferative cells. Therefore, molecularly targeted therapy increases the therapeutic window by only focusing on defined molecular abnormalities. The most prominent and well-explored targets are the growth factor receptors and downstream non-receptor kinase signaling. Targeting cancer metabolism, inhibitors of apoptosis and genetic modulation are also emerging strategies to inhibit cancer growth. One of the most successful targeted therapeutics is Gleevec, which is a kinase inhibitor. It has great affinity to the oncofusion protein BCR-Abl, which is a strong driver of tumorigenesis in chronic myelogenous leukemia. There are also targeted therapies for colorectal cancer, head and neck cancer, breast cancer, multiple myeloma,

lymphoma, prostate cancer, melanoma and others. Targeted therapies include monoclonal antibodies (mAbs), small molecule inhibitors and peptide mimetics. Monoclonal antibody therapy is a form of immunotherapy. The mAbs bind monospecifically to certain cells or proteins and thereby stimulate the patient's immune system to attack those cells. Most of the small molecules are tyrosine-kinase inhibitors and interfere with the binding and phosphorylation of the substrate of the respective tyrosine-kinase. Unfortunately, these molecularly targeted therapies only have drastic response rates in cancers that have a specific gene mutation. Therefore, only a small subset of patients will benefit from a molecularly targeted therapy whereas the response rates remain marginal across an unselected patient population [12].

1.3 Oxygen and its involvement in tumor treatment

Oxygen levels vary within normal tissue and between tumors. The amount of oxygen dissolved in blood is usually referred as partial pressure of oxygen: pO_2 [13].The physiological pO_2 in normal human tissues ranges from 20mmHg in liver and brain, to 70mmHg in kidney [14]. When the tissue oxygen level falls below physiological levels, the ability to sustain the normal cellular functions is impaired, leading to the emergence of pathologic states. This is often summarized as hypoxia. It is important to define hypoxia in relation to the oxygen dependence of biological processes, with the potential to influence disease behavior and treatment response [14]. Levels of oxygen of about 1% (= 5- 10mmHg) correspond to a hypoxic state, whereas levels above are referred to normoxic [15]. In general, pO_2 levels below 1% lead to an upregulation of hypoxia-inducible factors (HIFs). HIFs are transcription factors that mediate the response to decreases in available oxygen in the cellular environment [16].

In more than 50% of all cancer patients, but depending on the tumor entity, radiotherapy is applied [17]. Since the outcome of this treatment modality mainly depends on oxygen, tumor hypoxia represents a major challenge for successful radiotherapy [18].

The direct biochemical effect of oxygen on fixation of DNA damage (Figure 3), as a factor contributing to radiotherapy treatment outcome, is maximal at pO_2 levels above 5 mmHg [8]. Zones of pO_2 below this threshold are highly radio-resistant. Additionally, hypoxic cells are also more resistant to anti-cancer drugs in general because of several reasons;

First, hypoxic cells are more distant from blood vessels and are therefore less accessible for drugs. Second, the proliferation of cells decreases as a function of distance from blood vessels, an effect that is at least partially due to hypoxia. Third, hypoxic condition leads to the selection for cells that have lost sensitivity to p53-mediated apoptosis, which might lessen the sensitivity to some anti-cancer drugs. Fourth, low oxygen levels induce HIF-1α signaling, leading to upregulation of genes involved in drug resistance [8].

HIFs are the main players of the response to hypoxia. Among them is HIF-1α. The alpha subunits of HIF are hydroxylated at proline residues by HIF prolyl-hydroxylases. Through the hydroxylation, HIF-1α is recognized by the VHL E3 ubiquitin ligase, which labels it for the rapid degradation by the proteasome. The HIF prolyl-hydroxylase uses oxygen as a co-substrate. The hydroxylation functions therefore under normoxic conditions, but is abrogated under conditions with insufficient oxygen availability. When stabilized, HIF-1α acts as transcription factor and upregulates several genes to promote survival in low-oxygen conditions. The response includes the induction of angiogenesis through the expression of vascular endothelial growth factor (VEGF). To achieve the transition from aerobic to anaerobic metabolism, expression of the glucose-transporter1 (GLUT1) and expression of carbonic anhydrase IX (CA-IX) is increased. HIFs also regulate several other genes that promote vasodilation, apoptosis, autophagy, oxygen-sensing-invasiveness and metastasis [19]. Additionally, tumor cells repress processes in the cell that require higher amounts of energy, such as DNA repair, leading to an even more mutant phenotype [20]. Because of this, hypoxia is referred to select for a more malignant and invasive tumor, also by promoting metastasis by angiogenesis [21].

Consequently, reducing tumor hypoxia that can lead to tumor vessel normalization is a promising approach in cancer therapy [22].

Nonetheless, hypoxia can be exploited by using it for activation of prodrugs. Basically, hypoxia-selective cytotoxicity is mediated through a one-electron reduction of a non-operative prodrug, to a radical. The oxygen-dependent back-oxidation restores the original compound, thereby producing superoxide. Under hypoxic conditions, the back-oxidation does not take place and the prodrug radical accumulates. As it is more cytotoxic than the superoxide produced in normoxic cells, hypoxia-selective cell killing is achieved [23].

Hypoxia can also be exploited by hypoxia-selective gene therapy. The promotor which is responsive to HIF-1α is used to express proteins able to reduce these prodrugs [24]. Other approaches to exploit hypoxia in cancer therapy includes targeting HIF-1α directly [25]. Even recombinant anaerobic, genetically engineered, non-pathogenic bacteria, which only grow in the absence of oxygen, can be used [26].

In summary, hypoxia can be an aid in cancer therapy, using it as an activator for prodrugs, but unfortunately it also presents a main hurdle in general, as the prognosis for radio- and chemotherapy is worse in hypoxic tumors overall [8].

Hypoxia is a defining hallmark of malignancy. It arises from the high metabolic demands of rapidly proliferating cancer cells that rely on a dynamic, unpredictable and ineffective microvasculature. Hypoxia is not just a microenvironmental by-product of uncontrolled and poorly supported cellular growth. It is rather an active participant in the tumorigenesis and the evolution of a cancer over time.

1.4 The clinical significance of hypoxia

The progress in medical genomics and bioinformatics has led cancer research towards the development of targeted treatments, to focus on specific molecular or genomic susceptibilities. However, given the molecular complexity present in cancer, it became obvious, that highly specific targeted treatments only showed great success in a small subset of patients. Only by targeting the pathophysiological peculiarities related to specific signaling pathway can lead to a better utility of molecular treatment strategies.[27].

Hypoxia is one of those pathophysiological peculiarities, a mismatch of oxygen supply and demand. There is evidence for declaring hypoxia as one of the key determinants of cancer behavior and treatment outcome in patients. Although known, one has largely failed to implement this knowledge into clinical practice. There are several reasons for this, including the rather difficult identification of the optimal patient subgroups for a hypoxia-targeted treatment, the general intensification of the standard treatment over the same time frame and the complex interplay between hypoxia and other molecular determinants of treatment outcome [28]. The identification of such subgroups is of great importance, because hypoxia only has a major influence on treatment outcome when the patient shows a “hypoxia driver phenotype”. These patients comprise the population that is most likely to benefit from hypoxia targeted treatments [29].

The most intensely investigated clinical interest includes strategies to improve oxygen delivery to tumors: antiangiogenic treatment to decrease an abnormal vasculature structure, breathing hyperbaric oxygen, reduction of the cellular oxygen consumption (e.g. by metformin), sensitization of hypoxic cells to radiotherapy with hypoxia-targeting agents (e.g. 2-nitromidazole drugs such as nimorazole) or direct eradication of hypoxic cells (e.g. with hypoxia activated prodrugs such as evofosfamide) [30]. A summary of those attempts is presented in Figure 4.

Hypoxia not only decreases the impact of irradiation, it also drives the expression and activation of specific signaling cascades promoting survival pathways and angiogenesis and recruits cancer immune cells. [31]

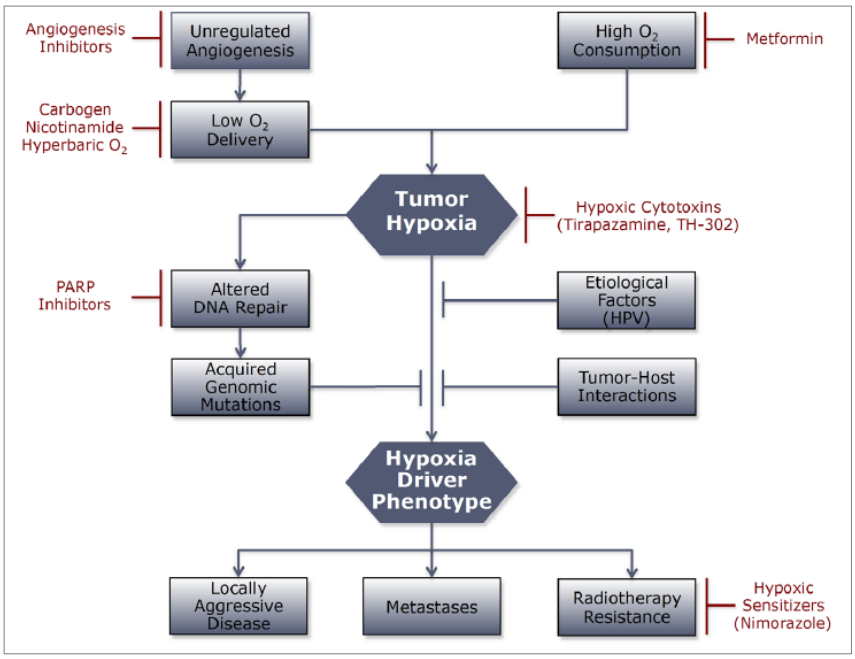


Figure 4 Summary of the factors influencing the impact of hypoxia on the clinical outcome. Hypoxia, even when present to the same extent in different tumors, may not have the same effect on clinical outcome because of a possible "hypoxia-driver phenotype". Figure adapted from [29].

1.5 Biomarkers for prediction of treatment response

Biomarkers are a possibility to assess and predict the response of cancer cells to an applied treatment. For radiotherapy, the initial sensitivity of cancer cells to irradiation is an important parameter to design and execute the irradiation schedule. To investigate the mechanics of tumor growth control upon ITPP treatment in combination with radiotherapy, biomarkers were essential to track the response of cancer cells [7].

1.5.1 γH2AX is a DNA double strand break marker

The intrinsic radiosensitivity relies on the capacity of the cell to repair DNA damage efficiently. Perturbed DNA repair mechanisms result in enhanced radiosensitivity. Among the different damages irradiation can induce, residual DNA DSBs present the highest lethal potential [32]. The histone variant H2AX gets rapidly phosphorylated upon a DNA DSBs, creating γH2AX (phosphorylated H2AX) at the chromatin regions that flank the DSB [33]. The phosphorylation of H2AX is a central event of the cellular response to DNA DSBs, because it serves as a docking site for various repair factors [34]. The number of γH2AX foci per cell is visualized with specific antibodies. It has been shown to correlate

with unrepaired DSBs, chromosomal aberrations and cell death [35]. The γH2AX foci form very rapidly after irradiation and reach plateau levels after 30 minutes. Thereafter, the number of foci decreases until they reach a residual level after 24 hours. After dephosphorylation H2AX, cells can re-enter the cell cycle by removing the checkpoint arrest [36]. For the mechanistic investigation, γH2AX- assay was used to assess the amount of initial and residual DNA DSBs upon ITPP treatment in combination with irradiation.

1.5.2 Pimonidazole as a hypoxia indicator

Since the oxygen content within a tumor highly influences the outcome of radiotherapy, the reporting of tumor hypoxia is crucial. Pimonidazole allows to histochemically assess hypoxia within different tissues and cell types. It is a 2-nitroimidazole that is reductively activated specifically in hypoxic cells at pO_2 levels below 1.3% (±10 mmHg). Once activated, it forms irreversible adducts with thiol groups in proteins, peptides and amino acids [37]. Pimonidazole visualizes therefore poorly oxygenated regions of histological sections and furthermore, the amount of pimonidazole that can be detected is proportional to the hypoxia within the tissue tested [38]. For these reasons, pimonidazole was used to identify hypoxic areas in tumor samples and to assess the differences upon ITPP treatment in combination with radiotherapy between poorly and well-oxygenated tumor fields.

1.6 Compound of interest: *Myo*-Inositoltrispyrophosphate

Myo-inositoltrispyrophosphate (ITPP) belongs to the family of *myo*-inositolphosphates; its basic structure consists of a cyclohexane ring: *myo*-inositol. Six phosphate groups are attached to the ring, two phosphates form a pyrophosphate through an ester-bond, resulting in three pyrophosphates.

ITPP was synthesized with 2,3-Bisphosphoglyceric acid (2,3-BPG) as a template. 2,3-BPG is an endogenous hemoglobin effector, that among other factors, controls the affinity of hemoglobin to oxygen. It binds the deoxygenated hemoglobin and decreases thereby the affinity to oxygen. 2,3-BPG allosterically promotes the release of the remaining oxygen molecules. Thus, it enhances the ability of red blood cells to release oxygen to cells that need it most, such as exercising cells [39].

Phosphatidylinositol-4,5-bisphosphate (PIP_2) and Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), two second messenger located at the plasma membrane, also contain *myo*-inositol as basic structure. For PIP_2 and PIP_3, two, respectively three phosphate groups are attached in addition to two long hydrocarbon chains which are essential for the location within the plasma membrane [40].

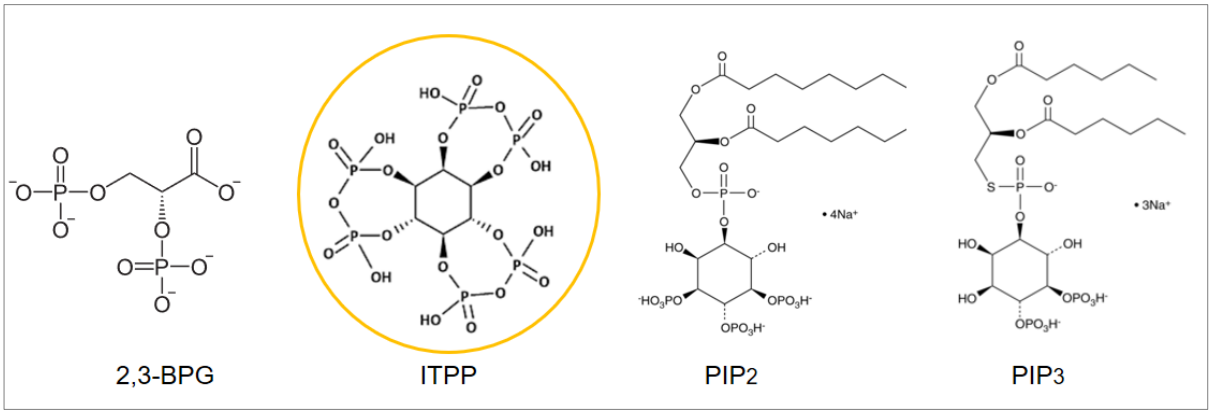


Figure 5 The structure of 2,3-BPG, ITPP, PIP_2 and PIP_3. Figures adapted from [40,41].

ITPP might interfere with two biological processes:

A) In an oxygen-dependent way: ITPP enters red blood cells via the band 3 anion transporter protein and thereupon reduces the affinity of hemoglobin to oxygen.

B) In an oxygen-independent way: ITPP is involved in the PI3K/AKT pathway since it presents similarities to PIP_2 and PIP_3.

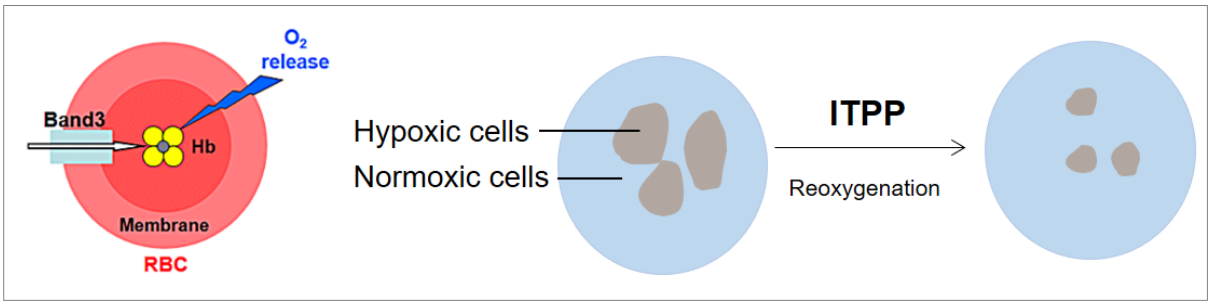


Figure 6 Scheme for reoxygenation upon ITPP treatment. ITPP enters red blood cells via band 3 anion transporter and reduces the affinity of hemoglobin to oxygen. Figure adapted from [59].

The effect of ITPP also has been previously investigated by other groups that demonstrated the ability of ITPP to selectively enter red blood cells via the band 3 anion transporter protein [59]. Also, the suppression of HIF-1α and eradication of early hepatoma in rats upon ITPP treatment was demonstrated [53]. Another group observed a stable tumor vessel normalization and increase of PTEN upon ITPP administration on the endothelial cell level [55].

1.7 PI3K/AKT pathway as a target for radiosensitization

The PI3K/AKT signaling pathway is one of the most critical pathways in the regulation of cell survival. The active serine-threonine protein kinase (AKT), also called protein kinase B (PKB), mediates cell proliferation, migration, angiogenesis and survival [41-43]. AKT contains two regulatory phosphorylation sites, Th308 in the activation loop within the kinase domain and Ser473 in the C-terminal regulatory domain [44]. The activation of AKT is mediated through Phosphatidylinositol-3 kinase (PI3K). Signaling of receptor tyrosine kinases upon binding of growth and survival factors activate PI3K in turn. PI3K phosphorylates PIP_2, which is located at the membrane. The generated PIP_3 recruits AKT to the plasma membrane, where activation steps through different kinases take place. PIP_3 levels are tightly regulated by the action of the Phosphate and tensin homolog phosphatase (PTEN). PTEN can remove a phosphate from PIP_3 and is therefore the main antagonist of the PI3K/AKT pathway and also one of the most frequently mutated tumor suppressors in cancer [45]. The activation of the PI3K/AKT pathway can lead to extensive VEGF secretion, both by HIF-dependent and independent mechanisms. Enriched levels of VEGF lead to the formation of abnormal vasculature, favoring the state of hypoxia [46]. To increase the radiosensitivity, the control of the PI3K/AKT pathway is a promising target. The correct regulation of the PI3K/AKT pathway leads to normalization of the vasculature and counteracts anti-apoptotic signaling.

Because of the similarity of ITPP to PIP_2, or PIP_3 respectively, and the importance of the PI3K/ATK pathway in cancer formation and progression, ITPP's involvement within this pathway also needed to be addressed.

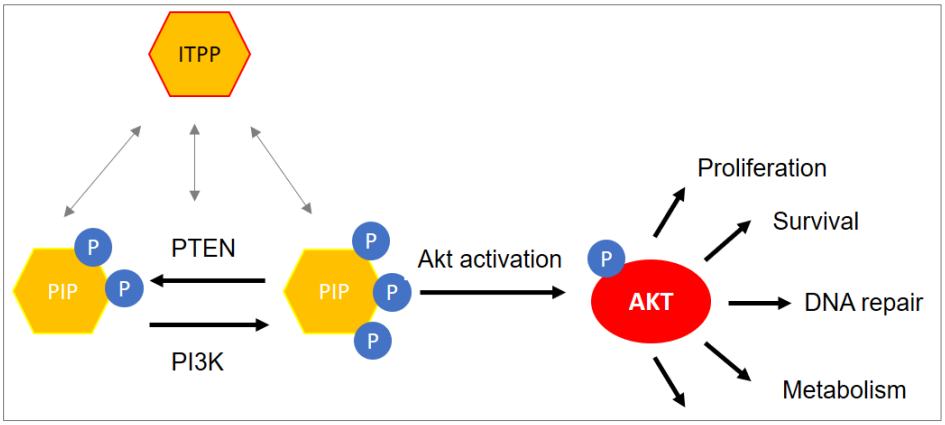


Figure 7 Simplified PI3K/AKT pathway. ITPP could interfere with different players within the pathway. Its detailed mode of action remains unrevealed. Figure adapted from [42].

1.8 Aim of the project

ITPP has previously been validated as an oxygen modulator by Ivo Grgic, PhD student in the Laboratory of Radiation Oncology at the University Hospital and tutor of this thesis. He demonstrated a significant increase in the oxygen concentration upon ITPP treatment within tumor xenografts derived from the lung adenocarcinoma cell line A549 (Supplementary 1A).

Furthermore - as ITPP presents a possible radiosensitizer through its hypoxia antagonizing role - an efficacy-study was recently carried out to determine the treatment impact of ITPP in combination with irradiation. In xenografted tumors derived from the FaDu head and neck squamous cell carcinoma cell line, which is less prone to form hypoxic tumors, a significant growth delay could be observed upon ITPP treatment in combination with irradiation (Figure 10A) [47].

Not all cancers have the same susceptibility for a certain treatment, which is influenced by several factors with hypoxia among them. Here, an additional cancer cell line, representing another subtype of cancer, was used to test the reoxygenation capability of ITPP and therefore its radiosensitizing effect.

This leads to the first part of this thesis:

A) **Can ITPP modulate the oxygen content within tumor xenografts derived from the head and neck cancer cell line FaDu, which forms less hypoxic xenografts compared to tumors derived from the non-small cell lung cancer cell line A549?**
B) **Can ITPP also act as a radiosensitizer in a highly hypoxic tumor like in xenografts derived from A549 cell line?**

The second and main part of the thesis is about mechanistic investigations of ITPP *in vivo*. These included investigations of the underlying cytotoxic, DNA-damage-oriented mechanism of irradiation when combined with ITPP.

C) **How does ITPP and subsequent reoxygenation quantitatively affect DNA damage?**

To investigate this part, a broad trial with histological staining for γH2AX was carried out to quantitatively assess initial and residual DNA DSBs upon irradiation in combination with ITPP, within hypoxic and normoxic areas of the same tumor.

Since ITPP has similarities to PIP_2 and PIP_3, *in vitro* investigation of ITPPs impact on the PI3K/AKT pathway was investigated as part of a side project.

D) **Which effect does ITPP have on the PI3K/AKT pathway in different (cancer) cell lines?**

In summary, this thesis aimed to investigate the capacity of ITPP to modulate hypoxia in different cell lines, and consequently, its radiosensitizing effect. Additionally, the effect on the PI3K/AKT pathway was investigated.

2 Material and Methods

2.1 Chemicals, Buffers and Solutions

Chemicals	
0.5 % Trypsin-EDTA 10x	Gibco by Life Technologies
2-mercaptoethanol	Sigma - Aldrich
Acrylamide	Sigma
Anti- Phospo-Akt (Ser473) Rabbit mAb	Cell Signaling
Anti- PTEN (138G6) Rabbit mAb	Cell Signaling
Anti β-Actin, Mouse mAb	Sigma Aldrich
Anti-PI 3 Kinase p110 alpha, Rabbit polyclonal IgG	Upstate Biotechnology
APS Ammonium Persulfate	BioRad
Bis-Acrylamide	BioRad
Blotting-Grade Blocker (Milk Powder)	BioRad
Bromphenol blue	Sigma - Aldrich
Coomaissie solution	0.25% Coomassie Brilliant Blue, 45% methanol, 10% acetic acid glacial
Coomassie Brilliant Blue	Sigma
Crystal violet	Merck
Dimethylsulfoxid (DMSO)	Sigma - Aldrich
DMEM Media	Gibco by Life Technologies
DNA Ladder 1 kb	New England BioLabs Inc.
ECL™ Anti-Mouse IgG Horseradish Peroxidase linked whole Antibody (from sheep)	GE Healthcare
ECL™ Anti-Rabbit IgG Horseradish Peroxidase linked $F(ab)_2$ fragment (from donkey)	GE Healthcare
ECL™ Western Blotting Detection Agents	Amersham™, GE Healthcare
EMEM Media	Gibco by Life Technologies
Endothelial Cell Basal Medium with addition of endothelial cell growth supplement (Heparin 0.004mg/ml, basic fibroblast growth factor (1mg/ml) and Hydrocortisone (1ug/ml)	PromoCell

Ethanol (EtOH)	Merck
Fetal Bovine Serum	Gibco by Life Technologies
Formalin	Kantonsapotheke Zürich
Glycerol	Sigma
Glyzine	Fluka
Hydrochlorid (HCl)	Sigma - Aldrich
Hygromycin	InvivoGen
Isoflurane	AbbVie, Baar
D-Luciferin	Perkin Elmer
Methanol (MeOH)	Morphisto
Myo- Inositoltrisphosphate (ITPP)	Normoxys
Paclitaxel	Sigma
PageRuler	Thermo Scientific
Paraffin	Merck
PBS pH 7.2	Kantonsapotheke Zürich
Pen Strep (Penicillin/Streptomycin)	Gibco by Life Technologies
Pimonidazole	HPI, Hydroxiprobe Inc.
Potassium Chloride (KCl)	Fluka Biochemika
Puromycin Dihydrochloride	Sigma
Polyvinylidene difluoride (PVDF) Transfer Membrane Amersham- P Hybond	GE Healthcare
RPMI Media	Gibco by Life Technologies
Sodium Chloride (NaCl)	Sigma-Aldrich
Sodium dodecly sulfate (SDS)	Sigma
Sodium Hydroxide(NaOH)	Honeywell
Tetramethylethylenamidin (TEMED)	BioRad
Trizma® base	Sigma
Tween® 20	Sigma

Table 1 Chemicals

Buffers and Solutions	
Acrylamide solution 30 %, 100 mL	• Acrylamide 29.2 g • Bis-Acrylamide 0.8 g • add 100 mL ddH_2O, sterile filtration, store at 4°C in aluminum foil
10 % APS	• 100 mg APS in 1 mL H_2O
Lämmli buffer	• 50 mM Tris HCl • 2% SDS • 10% Glycerol
NFMS	• 5% skimmed milk in TBS-T
SDS-sample buffer, 100 mL	• 125 mM Tris HCl pH 6.8 • 4 % SDS • 20 % Glycerol
SDS Loading Buffer	• 50 mM Tris-HCl pH 6.8 • 2% SDS • 10% glycerol • 1% β-mercaptoethanol • 0.02 % bromophenol blue
Running Gel buffer	• 1.5 M Tris, pH 8.8 • 0.1 % SDS
Stacking Gel buffer	• 0.5 M Tris, pH 6.8 • 0.1 % SDS
Transfer buffer	• 25mM Tris HCl • 192mM Glycine • 0.1% SDS • 20% MeOH
TBS (10 x)	• NaCl 80 g • KCl 2 g • Tris 30 g • add 1 L ddH_2O, pH 7.4
TBS-T	• 1 X TBS + 0.1% Tween20

Table 2 Buffers and Solutions

2.2 *In vitro*: assessment on the effect of ITPP on different cell lines

2.2.1 Cell culture

The human squamous head and neck cancer cell line FaDu was cultured in EMEM cell culture media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). The human umbilical vein endothelial cell line HUVEC was cultured in ECBG cell culture medium supplemented with endothelial cell growth media. The non-small cell lung carcinoma cell line A549 was cultured in RPMI1640 cell culture medium, supplemented with 10% FBS and 1% P/S. The cells were cultured as monolayers in culture plates in a 37°C humidified atmosphere containing 5% CO_2 (Binder incubator). Cell culture work was conducted once to twice a week. For HUVECs, bio-coated cell ware was used.

ITPP was provided by NormOxys, dissolved in PBS and further diluted with cell culture media for treatment.

Cell lines		
A549	Human non-small cell lung carcinoma	ATCC® CCL-185™
FaDu	Human head and neck squamous cell carcinoma	ATCC® HTB-43™
HUVEC	Human umbilical vein endothelial cells	ATCC® CRL-1730™

Table 3 Cell lines

2.2.2 Irradiation of cells

Cells were irradiated with 0, 2, 4 and 6 Gy using an Xstrahl 200 kV X-ray unit at 1 Gy/min (Gulmay, Suwanee GA).

2.2.3 Western Blotting

For sample preparation, media was removed from the petri dishes and cells were washed once with PBS. For harvesting, the cells were scraped off in Lämmli Buffer and heated for 5 min at 95°C, shaking at 1000 rpm (Thermomixer compact, Eppendorf). The protein concentration was determined by Nanodrop 1000 (Spectrophotometer, Thermo Scientific).

Samples were stored at -20°C.

Protein samples (50µg) from whole cell lysates were size separated by SDS-PAGE in 10% acrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer unit (TE70X, Hoefer). A PageRuler prestained protein ladder was used as a marker.

- Running gel: 10%: 30% acrylamide 5ml, running gel buffer 3.75ml, H_2O 6.25ml, TEMED 10µl, 10% APS 100µl

- Stacking gel: 30% acrylamide stock 1.3ml, stacking gel buffer 2.5ml, H_2O 6.1ml, TEMED 10µl, 10% APS 50µl

After the transfer, membranes were blocked in 5% non-fat milk solution (NFMS) for 1h at room temperature before overnight incubation at 4°C with different primary antibodies (1:1000 in 5% NFMS). Before and after membranes were probed for primary antibody with anti-rabbit (1:2000) and anti-mouse (1:2000) ECL peroxidase conjugates, they were washed three times for at least 15 min with TBS-T. The resulting chemiluminescent complexes were identified by ECL™ Western Blotting detection agents by a Vilber Lourmat Fusion FX Detector. Images were stored as TIF files for analysis.

2.2.4 *In vitro* ITPP treatment

Per treatment, 200'000 cells were seeded in 2ml medium and allowed to grow and adhere overnight. The cells were treated with 10mM ITPP for different time periods (2h, 6h, 24h, 48h) and cultured in either hypoxic or normoxic condition.

- Hypoxic condition: 37°C, 5% CO_2, 0.2% O_2, in a humidified Scholzen incubator
- Normoxic condition: 37°C, 5% CO_2, 19% O_2, in a humidified Binder incubator.

After treatment, cells were harvested for Western Blot analysis (3.2.3).

2.2.5 Proliferation assay

For the proliferation assay, transparent 96-well plates were used. The outer rows were filled with PBS to prevent evaporation. For every timepoint (0h, 24h, 48h, 72h) and every condition, triplets were performed. The proliferative activity was measured by the capacity of the cells to metabolize AlamarBlue (Biosource International, Camarillo, CA). Through metabolization within the cell, AlamarBlue is irreversibly reduced to the pink colored and highly red fluorescent resorufin. The change in absorbance can be measured and indicates the proliferative activity. Exactly 3h prior measuring the absorbance, 10µl AlamarBlue was administered to the corresponding wells. The absorbance at 590nm and 630nm was measured by the Tecan GENios spectrophotometer.

2.2.6 Clonogenic cell survival assay

To investigate the ability of cells to form colonies, clonogenic cell survival assays were performed. Cells were seeded in petri dishes to obtain ± 50 colonies. Cells were preincubated with 10mM ITPP for different time periods (2h, 6h and 24h) and irradiated with 0, 2, 4 and 6 Gy using an Xstrahl 200 kV X-ray unit at 1 Gy/min (Gulmay, Suwanee GA). Cells were allowed to form colonies for at least 15 days. For fixation of the colonies, the media was removed, and the cells were washed once with PBS. The cells were incubated for 1-2 h with methanol/acetic acid 3:1. After removal of the methanol/acetic acid mixture, the plates were left to dry overnight at room temperature (RT). Afterwards, the colonies were

stained by overlaying the plates with crystal violet for 30 min. The staining mixture was removed by gentle washing with tap water and the plates were left to dry overnight at RT. For the evaluation of the assay, the colonies formed were counted manually with a colony counter (Gallenkamp). The plating efficiency (PE) was calculated by dividing the number of colonies by the cells seeded on the control plate (here 0 Gy). The survival fraction (SF) could be calculated the following way: $SF=\frac{\text{Colonies counted}}{\text{Cells seeded}/_{PE}}$ [3].

2.3 *In vivo* assessment of ITPP impact

All in *vivo* experiments were performed according to the guidelines of welfare and use of animals of the Veterinäramt Zurich, Switzerland.

2.3.1 Animals

Female athymic CD1 nude mice (Charles River) were kept in appropriate conditions.

2.3.2 Subcutaneous injection for xenograft generation

For each xenograft, 4×10^6 cells (in 250µL PBS) were injected subcutaneously in the flank of athymic CD1 nude mice under anesthesia with Isoflurane. Tumor growth was measured and determined from Caliper skin measurements of tumor length (L) and width (l) according to the formula $\frac{L\times l^2}{2}$. Tumors were allowed to expand to a volume of $300mm^3$ (±10%) before treatment start and random assignment of animals to the experimental groups.

2.3.3 ITPP treatment and pimonidazole injection

3g/kg bodyweight ITPP was injected intraperitoneally (i.p.). ITPP was dissolved in PBS and pH was adjusted to physiological pH using 1M NaOH.

60mg/kg bodyweight pimonidazole was injected i.p. 4h before treatment start. Pimonidazole was dissolved in 0.9 % NaCl solution.

2.3.4 Dynamic *in vivo* imaging system: IVIS

IVIS is a Living Image system by Perkin Elmer, enabling to acquire and analyze luminescent or fluorescent signals in real-time and it provides tools for optimizing image display and analyzing images or kinetic data. The Living Image 3.2 software was used to obtain bioluminescent pictures [48] (Caliper Life Sciences). Luminescence signal was measured right before ITPP treatment as a baseline and corrected for tumor volume.

To observe the capacity of ITPP to be an oxygen- modulator, an *in vivo* bioluminescence reporter system was used.

In brief, a part of the oxygen dependent degradation (ODD) domain of HIF-1α is fused to luciferase and constantly expressed in the cancer cell line of interest. Under normoxia, the ODD domain is hydroxylated by HIF prolyl-hydroxylase at two proline residues, leading to the degradation of HIF-1α protein by the proteasome. Under hypoxia, the proline is not hydroxylated, leading to stabilization of the protein and expression of luciferase. This can be used as a reporter for hypoxia under low oxygen concentration. The bioluminescence signal is increased compared to normal oxygen concentrations because of the stable expression of luciferase, which can convert the injected luciferin into a measurable bioluminescent signal.

When sufficient oxygen is present, the luciferase is rapidly degraded due to its fusion to the ODD domain. Therefore, a strong bioluminescent signal upon luciferin application represents hypoxia, whereas a decrease in the luminescent signal refers to an increase in oxygen [49].

To test the oxygen-modulating capabilities of ITPP, tumor xenografts derived from FaDu cell line in athymic CD1 nude mice were used.

Treatment started when the tumor reached a volume of 300mm^3 ± 10% and animals were randomly assigned to an experimental group. Prior to the injection of ITPP (3g/kg bodyweight) or PBS as a control, a baseline of the bioluminescence signal was acquired. 2h after the treatment injection, the first bioluminescent measurement was taken. ITPP was applied on two consecutive days. The dose- and time window for maximal ITPP effect was already identified in a previous project, in another tumor model [47].

2.3.5 PXi and SmART for irradiation design and execution

The precision X-ray X-RAD 225Cx (PXi) machine is a small animal image guided irradiation system. It has the feature to acquire optical images in addition to cone beam CT (CBCT) scans. Pilot X-ray software 1.14.1 was used to scan the tumors and execute irradiation treatment. To individually design a treatment, the planning software SmART-Plan, version 2.0 November 2014, was utilized [50][51]. With the PXi, maximal target accuracy for irradiation was ensured. An example for a treatment plan is presented in Figure 8.

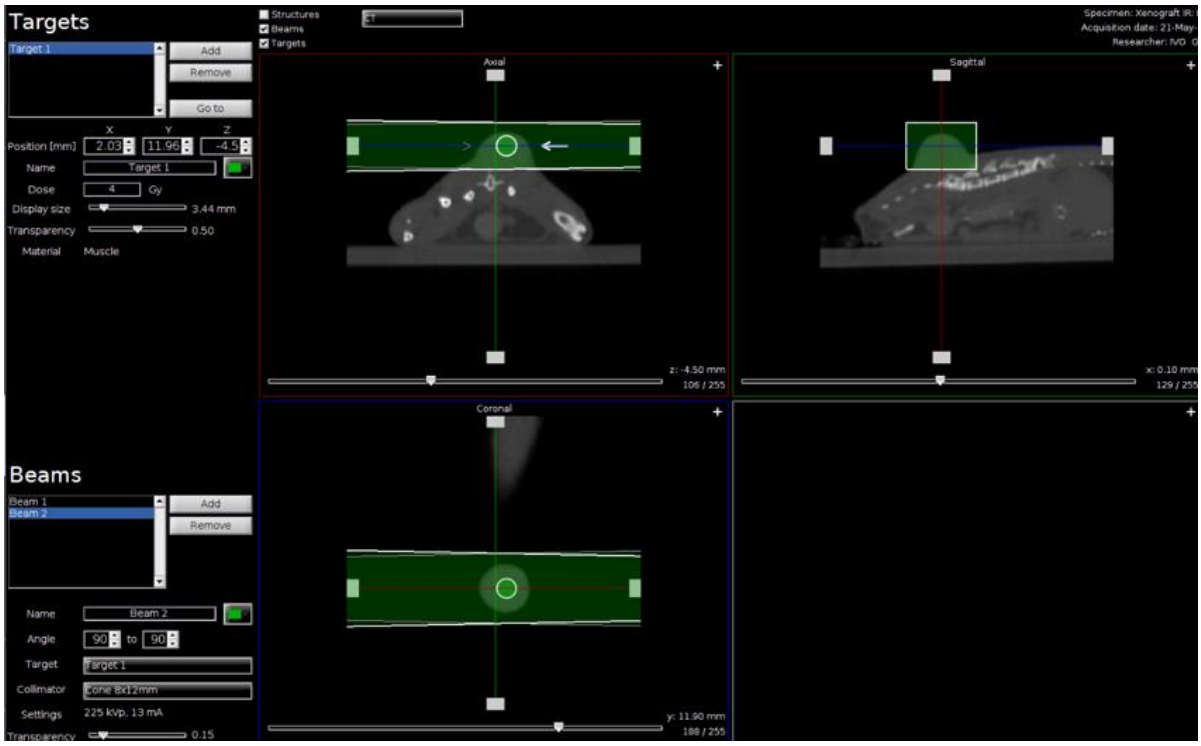


Figure 8 Example of a SmART treatment plan for xenograft irradiation using PXi. CT scans from sagittal, coronal and axial view. The irradiation beam is presented in green.

2.3.6 γH2AX assay

A549-derived xenografts were generated as described in 2.3.2. Treatment started at a tumor volume of 300mm^3 ± 10%. 4h before treatment, pimonidazole was injected i.p. at 60mg/kg bodyweight. Treatment was applied on two consecutive days and eventual irradiation with 4 Gy took place 2h after the second injection. The tumor was stored in formalin immediately after excision and embedded into paraffin 24h thereafter. In contrast to the efficacy-oriented experiment with FaDu-derived xenografts (Figure 10A), irradiation intensity for the γH2AX assay was chosen to be 4 instead of 5 Gy to avoid induction of too many γH2AX foci per cell, ensuring proper counting of single γH2AX foci [34].

Three consecutive 3µm specimen cross-sections from the paraffin-embedded tumor material were stained for

a) hematoxylin and eosin (H&E),

b) pimonidazole

c) and γH2AX

The preparation and staining of tumor sample slides was carried out by SophistoLab, Muttenz and the scanning by a digital color camera in the Pathology Department of the University Hospital Zurich.

For the evaluation, three hypoxic zones (pimonidazole-positive staining) and three normoxic zones, were chosen for each tumor sample and γH2AX foci were counted manually in at least 100 cells in the defined zones.

Foci analysis was performed in intact and viable cell nuclei only. Mitotic, necrotic or cells that showed micronuclei were excluded from the analysis. This was decided upon H&E staining. Number of foci was normalized to nucleus volume using ImageJ.

2.4 Statistical analysis

Statistical analysis was performed with GraphPad Prism 7. Data is represented as mean ± SEM. The data was analyzed using student t test, Mantel-Cox test or one-way Anova.

$P \leq 0.05$ was considered significant.

3 Results

3.1 *In vivo* assessment of ITPP as a combined treatment with radiotherapy

3.1.1 ITPP acts as a hypoxia modulator

In order to assess tumor reoxygenation in response to ITPP treatment, an *in vivo* bioimaging approach was used, in which the oxygen degradation domain (ODD) of HIF-1α is fused to luciferase and expressed in the cell line of interest, in this case FaDu head and neck squamous cell carcinoma cells. The effect of ITPP was tested in FaDu-derived xenografts growing in athymic CD1 nude mice, to complement the experimental series of Ivo Grgic, who performed the reoxygenation-experiment already in A549-derived xenografts and carried out an efficacy-study in FaDu-derived xenografts (Figure 10A) [47]. Treatment started at a tumor volume of 300mm^3 ±10% and animals were randomly assigned to an experimental group. ITPP or PBS was injected on two consecutive days. 2h after each injection, the bioluminescence signal was measured and normalized to a baseline bioluminescence signal and tumor volume.

A significant decrease in tumor bioluminescence was detected 2h after the second ITPP treatment (Figure 9B), indicating a significant increase of the oxygen concentration within the tumor ($p=0.03$, student t-test), as compared to tumors treated with PBS (Figure 9A). The same effect was previously observed in A549-derived xenografts; The decrease in hypoxia was already significant 2h after the first ITPP injection (Supplementary 1A) [47].

Collectively, this result indicates that ITPP can significantly decrease hypoxia in A549- and FaDu-derived xenografts in athymic CD1 nude mice.

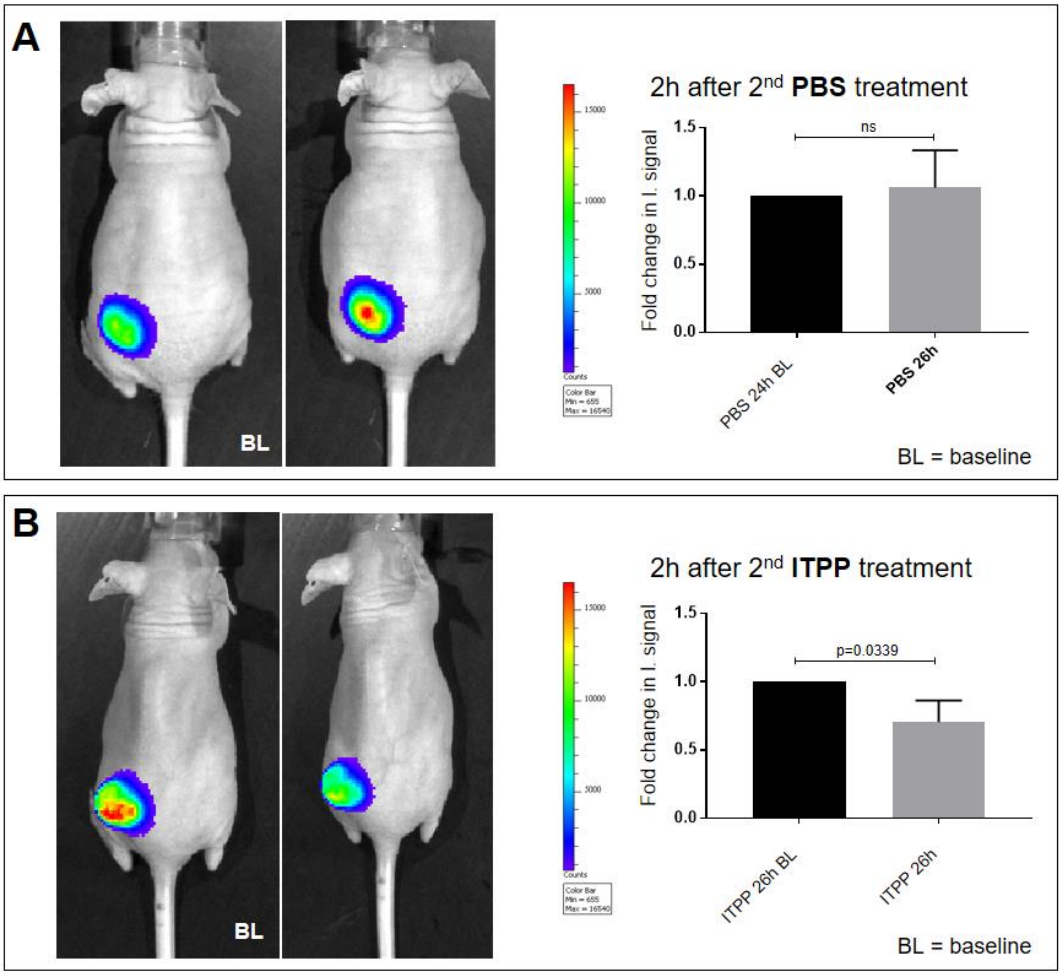


Figure 9 Reoxygenation of FaDu-derived xenografts upon ITPP treatment: representative images illustrating the effect on ITPP treatment as compared to control treatment on tumor reoxygenation, detected by a hypoxia-specific bioluminescent *in vivo* bioimaging approach. **A)** For control treatment, there is no significant change in luciferase activity detectable within the FaDu-derived xenograft. **B)** 2h after the second ITPP treatment, there is a significant decrease in luminescent signal observable, indicating an increase in oxygen.

3.1.2 ITPP in combination with irradiation leads to a significant growth delay in tumor xenografts

The results obtained with the *in vivo* bioluminescence imaging system suggest, that ITPP treatment with a subsequent increase in tumor oxygenation might sensitize for irradiation [3]. Irradiation acts upon formation of ROS that can induce DNA damage. This damage can be further stabilized through additional ROS. For the fixation of DNA damage, oxygen is required [4]. In theory, by lowering tumor hypoxia, the response to irradiation is more effective in a reoxygenated tumor as compared to a hypoxic tumor. Oxygen enhancement ratios for up to 2.5 to 3 were identified [3].

To test the effect of ITPP as a "reoxygenator" and therefore its capacity to act as radiosensitizer, A549-derived xenografts in athymic CD1 nude mice were treated with a single fraction of irradiation alone and in combination with ITPP. Treatment started at a tumor volume of 300mm^3 ±10% and animals were randomly assigned to an experimental group. ITPP or PBS respectively was injected on two consecutive days. The xenografts were irradiated with 5 Gy 2h after the second treatment, using an image-guided small animal radiotherapy platform (XRAD 225Cx, precision X-ray). The radiotherapy platform is linked to the SmART planning software. With this software, a detailed treatment plan could be designed and executed. Maximal target accuracy was ensured (2.3.5). The treatment schedule outline was based on the previous efficacy-study within FaDu-derived xenografts treated with ITPP in combination with irradiation. This experiment was performed by Ivo Grgic (Figure 10A). The experiment was conducted in xenografts derived from the head and neck squamous cell carcinoma (HNSCC) cell line FaDu because of the interest claimed by NormOxys and the clinical relevance of HNSCC. A significant tumor growth delay upon combination treatment of ITPP with irradiation was demonstrated ($p=0.03$, student t-test). The tumor growth delay upon irradiation in combination with ITPP was also significant in A549-derived xenografts as compared to irradiation treatment alone ($p=0.05$, student t-test) (Figure 10B). Monotreatment of ITPP did not exert in any tumor growth delay as compared to control treatment (PBS).

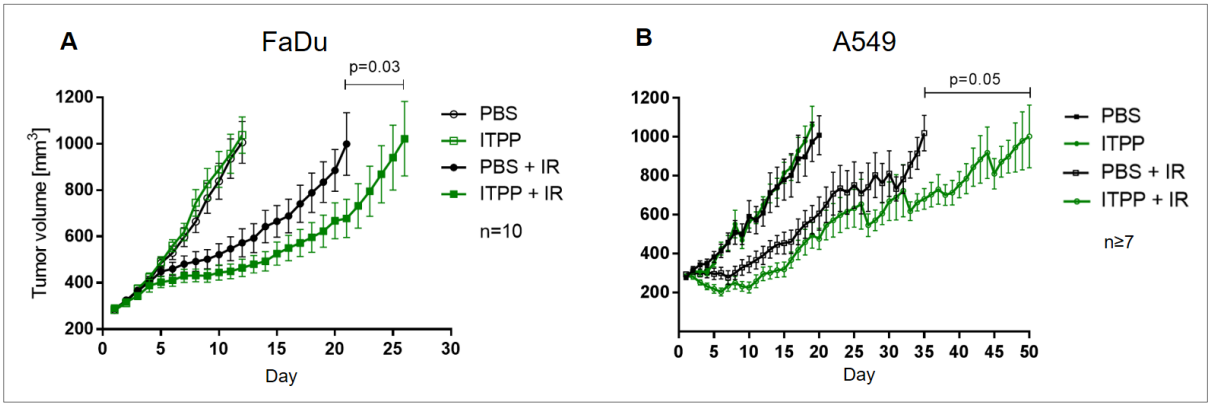


Figure 10 Tumor growth delay upon ITPP treatment in combination with irradiation. **A)** FaDu-derived xenografts were irradiated with 10 Gy (experiment performed by Ivo Grgic). **B)** A549-derived xenografts were irradiated with 5 Gy. Monotreatment with ITPP did not have any effects on tumor growth of FaDu- and A549-derived xenografts.

Furthermore, the absolute growth delay to quadruple the initial tumor volume (300 mm^3) of A549-derived xenografts was most enhanced in response to the combined treatment modality when compared with the absolute tumor growth delay in response to irradiation alone. Median survival for treatment with ITPP in combination with irradiation was 78d and the irradiation only average survival was 42d. The survival was significantly prolonged (p=0.02, one-way Anova) for combined treatment modality of ITPP and irradiation (Figure 11).

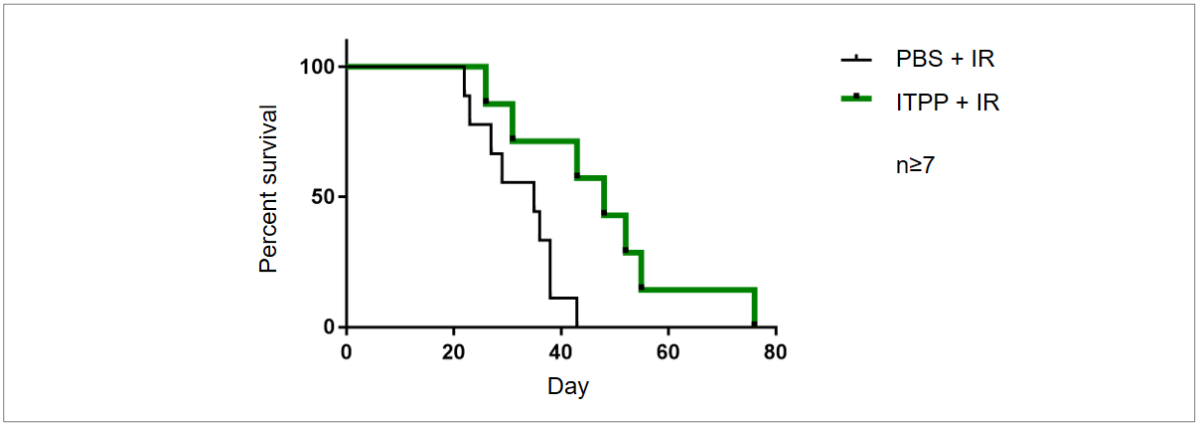


Figure 11 The median survival of mice bearing A549-derived xenografts upon combination treatment with ITPP and irradiation was significantly prolonged as compared to mice bearing A549-xenografts that received radiotherapy only.

The results obtained from the efficacy-studies in xenografts derived from A549 and FaDu cell line indicate that ITPP in combination with irradiation can lead to a significant tumor growth delay and prolonged survival.

3.1.3 Mechanistic investigation of ITPP in combination with irradiation shows significant increase in number of DNA DSBs within initially hypoxic areas

In order to complement the previously performed efficacy-oriented experiments in FaDu-derived xenografts, the effect of ITPP in combination with irradiation was assessed on the level of the DNA DSB induction using the DSBs marker γH2AX. To do so, FaDu-derived xenografts were treated with PBS, ITPP and irradiation (4 Gy) alone and combined. Treatment (3g/kg bodyweight ITPP/PBS) was injected on two consecutive days, and tumors were irradiated 2h after the second treatment injection. When the tumor xenograft reached a volume of 300mm^3 ± 10%, pimonidazole was injected i.p. at 60 mg/kg bodyweight in order to mark the hypoxic areas within the tumor. 4h thereafter, treatment with ITPP or PBS was initiated with animals randomly assigned to the different experimental groups. Tumors were harvested for histological characterization at a short term timepoint (0.5h after irradiation) or at a later timepoint (24h after irradiation). Directly after excision, tumors were stored in formalin and 24h later embedded in paraffin. Preparation of histological slides was carried out by SophistoLab, Muttenz. For each sample, three normoxic and three hypoxic zones were identified and in each zone, γH2AX foci of at least 100 cells were counted.

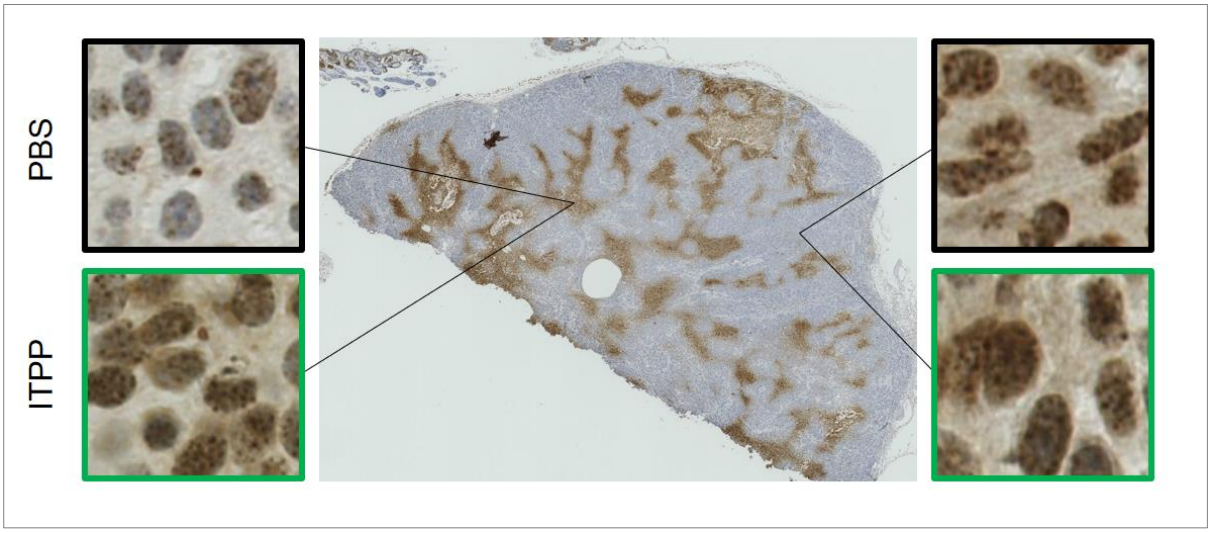


Figure 12 Representative tumor-histology slide stained for pimonidazole and γH2AX. γH2AX appears as brown foci within the cell nucleus. The squares represent samples within hypoxic zones (pimonidazole-positive: brown, left) or normoxic zones (pimonidazole-negative: blue, right), with one representative picture of each treatment (ITPP and PBS in combination with irradiation).

The relative frequency of γH2AX foci per cell in normoxic zones for unirradiated xenografts did not vary for either ITPP or control treated animals for any timepoint. 20 – 25% of the cells did not show any γH2AX foci at all (Figure 13A).

The hypoxic zones within unirradiated xenografts present a similar number of γH2AX foci per cell as cells within normoxic, unirradiated fields. There is no significant difference between ITPP treated and PBS treated hypoxic zones in regard to the average number of γH2AX foci per cell (Figure 13B).

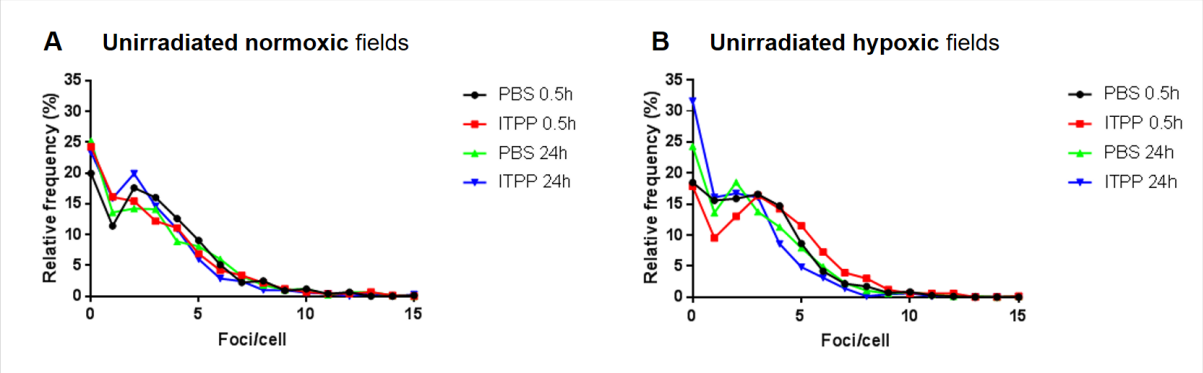


Figure 13 Relative frequency of cells with different numbers of γH2AX foci in unirradiated xenografts within **A)** normoxic cells and **B)** hypoxic cells. There was no difference in average γH2AX foci per cell for either ITPP nor PBS treatment at any timepoint.

Looking at the short term timepoint (0.5h) after irradiation, there were differences between hypoxic and normoxic zones.

The number of γH2AX foci per cell within the normoxic zones is similar for ITPP monotreatment and control treatment. Nevertheless, there are slightly more γH2AX foci per normoxic cell upon ITPP treatment combined with irradiation at the short term timepoint (0.5h after irradiation) (Figure 14B).

Shortly after irradiation, hypoxic cells had in average significantly more γH2AX foci per cell when treated with ITPP as compared to control irradiated hypoxic cells ($p=0.0006$, student t-test) (Figure 14A). This resulted in a right shift within the distribution curve of average γH2AX foci per cell. Nearly 0% of hypoxic ITPP treated cells had zero foci whereas 10% of hypoxic control irradiated cells had no γH2AX foci (Figure 14A right).

Also, for the long term timepoint after irradiation (24h), cells that were hypoxic prior ITPP treatment had more γH2AX foci per cell in average, as compared to hypoxic cells that were only treated with irradiation ($p=0.0001$, students t-test). 15% of the control irradiated hypoxic cells had zero γH2AX foci per cell, whereas for hypoxic cells treated with ITPP and irradiation, only 5% had no γH2AX-foci (Figure 14C).

Interestingly, irradiated normoxic cells also had more γH2AX-foci per cell at the long term timepoint, when treated with ITPP as compared to control irradiated cells, but almost 15% of control irradiated cells had no γH2AX-foci whereas upon ITPP treatment, only 5% of the cells had no foci (Figure 14D).

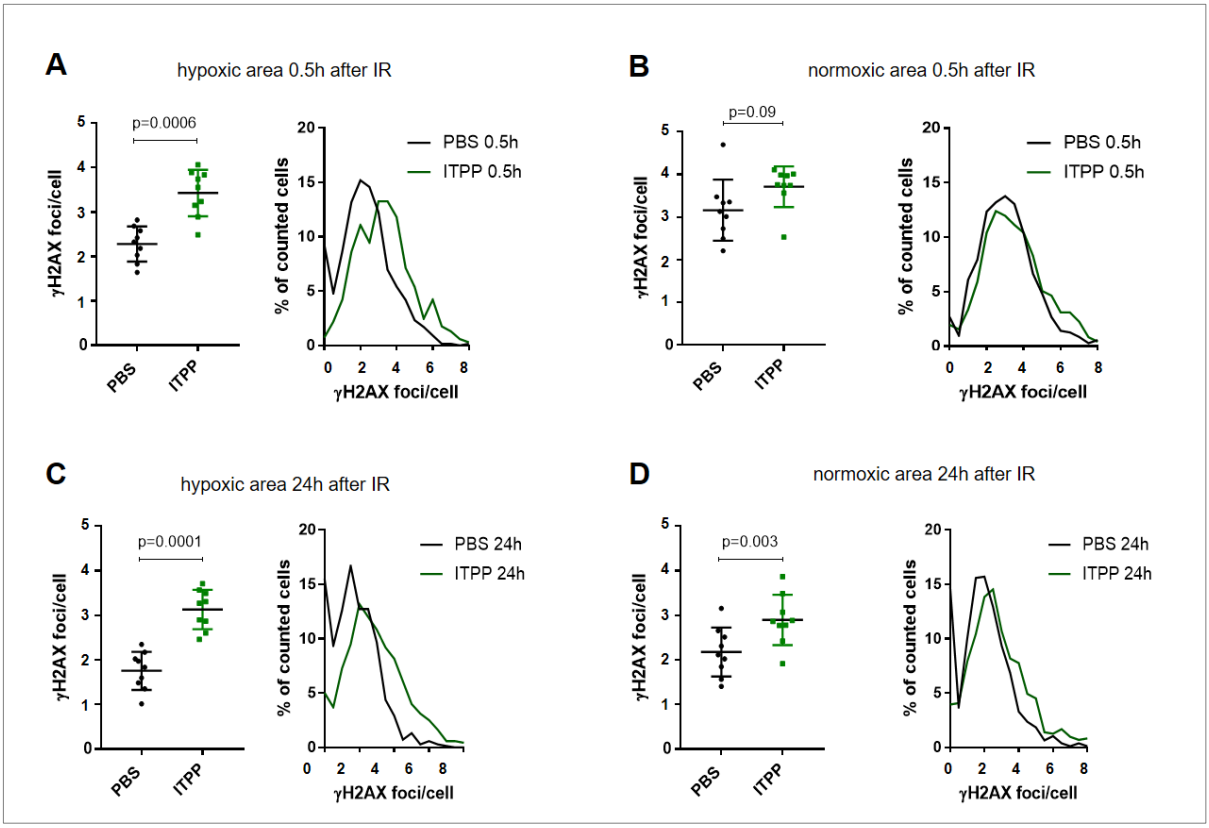


Figure 14 γH2AX foci per cell in irradiated xenografts**. A)** 0.5h after irradiation initially hypoxic cells (pimonidazole-positive) had in average more γH2AX foci per cell as compared to hypoxic cells that only received irradiation. 0% of ITPP treated and irradiated initially hypoxic cells had zero foci, whereas 10% of control irradiated hypoxic cells had no foci. **B)** 0.5h after irradiation, normoxic cells that were ITPP treated had slightly more γH2AX foci per cell. **C)** At a long term timepoint after irradiation (24h), there were overall less γH2AX foci per initially hypoxic cell. ITPP treated and irradiated cells still presented more γH2AX foci per cell in average and less cells with no γH2AX foci. **D)** For the long term timepoint after irradiation, normoxic ITPP treated cells still presented more γH2AX foci per cell as compared to control irradiated normoxic cells.

Overall, these results suggest that in initially hypoxic cells (pimonidazole-positive) treated with ITPP in combination with radiotherapy, more γH2AX foci per cell were induced, compared to hypoxic cells that only received irradiation treatment. Normoxic cells treated with ITPP and radiotherapy also have in average more γH2AX foci per cell, compared to normoxic cells that were only irradiated.

3.2 *In vitro* assessment of ITPP acting on the PI3K/AKT signaling pathway

Given the importance of the PI3K/AKT pathway in tumorigenesis and progression, the effect of ITPP was assessed within this pathway. By Western Blotting, protein levels of the key players within the PI3K/AKT pathway were assessed upon ITPP treatment. These experiments were performed as a side project and have not been carried out to full extent. Nevertheless, some of the results were included in this thesis.

3.2.1 ITPP-dependent increase of PTEN in normoxia and hypoxia

To assess the impact of ITPP on the PI3K/AKT pathway, Western Blots of whole cell lysates were performed and probed for different proteins within the PI3K/AKT pathway upon ITPP treatment. Cells were incubated for different time periods (2h, 6h, 24h and 48h) with 10mM ITPP or PBS. Cells were cultured in either normoxic or hypoxic conditions. The Western Blot was probed for PTEN, PI3K, pAKT and β-actin. AKT was not probed in this experiment because only the active form (pAKT) was of interest.

For the A549 cell line, an ITPP-dependent increase in PTEN was observed at the 24h time point (Figure 15A). Although there was also an increase in PTEN observed in PBS treated A549 cells over time (maximal after 48h), the decrease in pAKT was only present upon ITPP treatment. Within the FaDu cell line, an ITPP-dependent increase in PTEN was observed over time and simultaneously a decrease in pAKT level (Figure 15B).

Within hypoxic condition, the increase in pAKT was abolished upon ITPP treatment in A549 cell line, in comparison to A549 cells treated with PBS (Figure 15C). The hypoxia-dependent increase in pAKT was also demonstrated in FaDu cell line treated with PBS, but upon ITPP treatment, a decrease in pAKT was observed. Concomitantly, a slight increase in PTEN was present in FaDu cell line upon ITPP treatment (Figure 15D).

A similar response to ITPP treatment was demonstrated in HUVECs (data not shown).

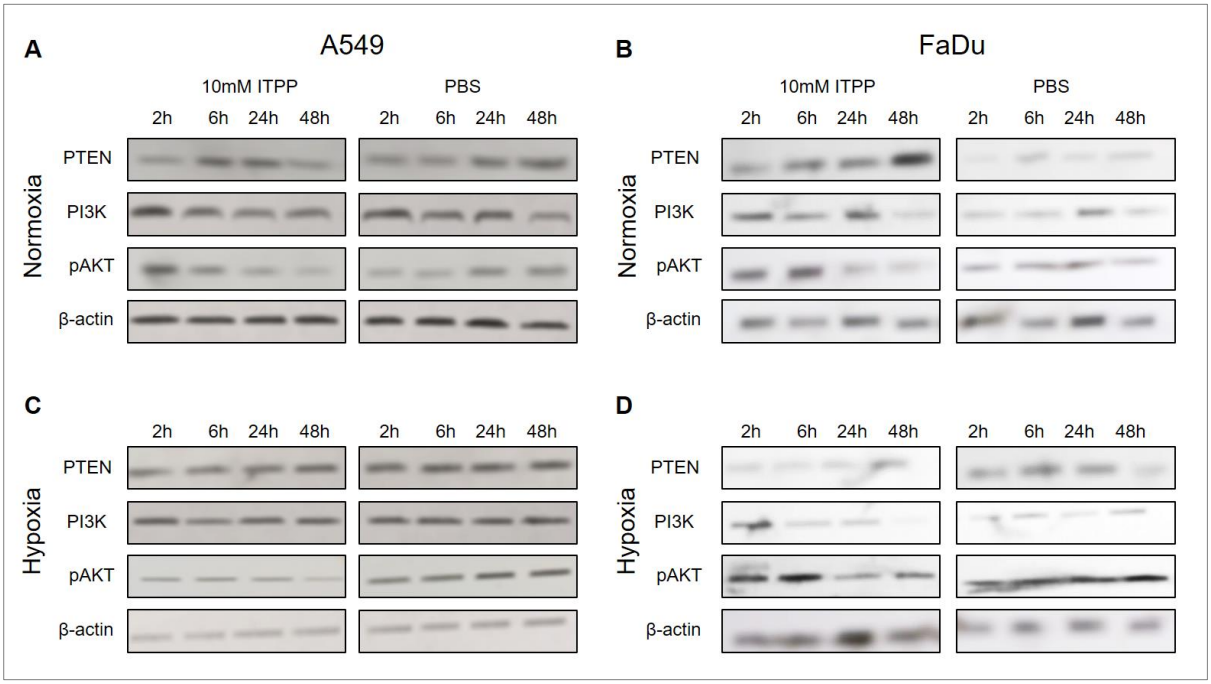


Figure 15 Representative Western Blots probed for different proteins within the PI3K/AKT pathway derived from A549 (left) and FaDu (right) whole cell lysates. The cells were incubated for different time periods with 10mM ITPP or PBS in normoxic or hypoxic conditions. **A)** A decrease in pAKT level was demonstrated in an ITPP-dependent manner in normoxic conditions in A549 cell line. **B)** An ITPP-dependent increase in PTEN was demonstrated in FaDu cell line and a concomitant decrease in pAKT level. **C)** Under hypoxic conditions, the hypoxia-dependent increase in pAKT level was abolished upon ITPP treatment in A549 cell line. **D)** The ITPP-dependent decrease in pAKT level under hypoxic conditions was also observed in FaDu cell line.

3.2.2 ITPP-dependent increase of PTEN upon combination with irradiation

In order to assess the response in cells treated with ITPP in combination with irradiation, FaDu cells and HUVECs were seeded and treated with 10mM ITPP 2h prior to irradiation with 2 Gy. Protein levels were assessed at different timepoints after irradiation (0.5h, 4h and 24h) by Western Blotting. The Western Blot was probed for PTEN, PI3K, pAKT and β-actin. AKT was not probed in this experiment because only the active form (pAKT) was of interest.

Upon irradiation, an ITPP-dependent increase in PTEN and a simultaneous decrease in pAKT level was demonstrated in FaDu cell line (Figure 16A) compared to FaDu cells that only received radiotherapy in which PTEN level decreased. In HUVECs treated with ITPP in combination with irradiation, the ITPP-dependent increase in PTEN was also demonstrated and the concurrent decrease in pAKT was observed (Figure 16B).

A similar response to ITPP in combination with irradiation was demonstrated in A549 (data not shown).

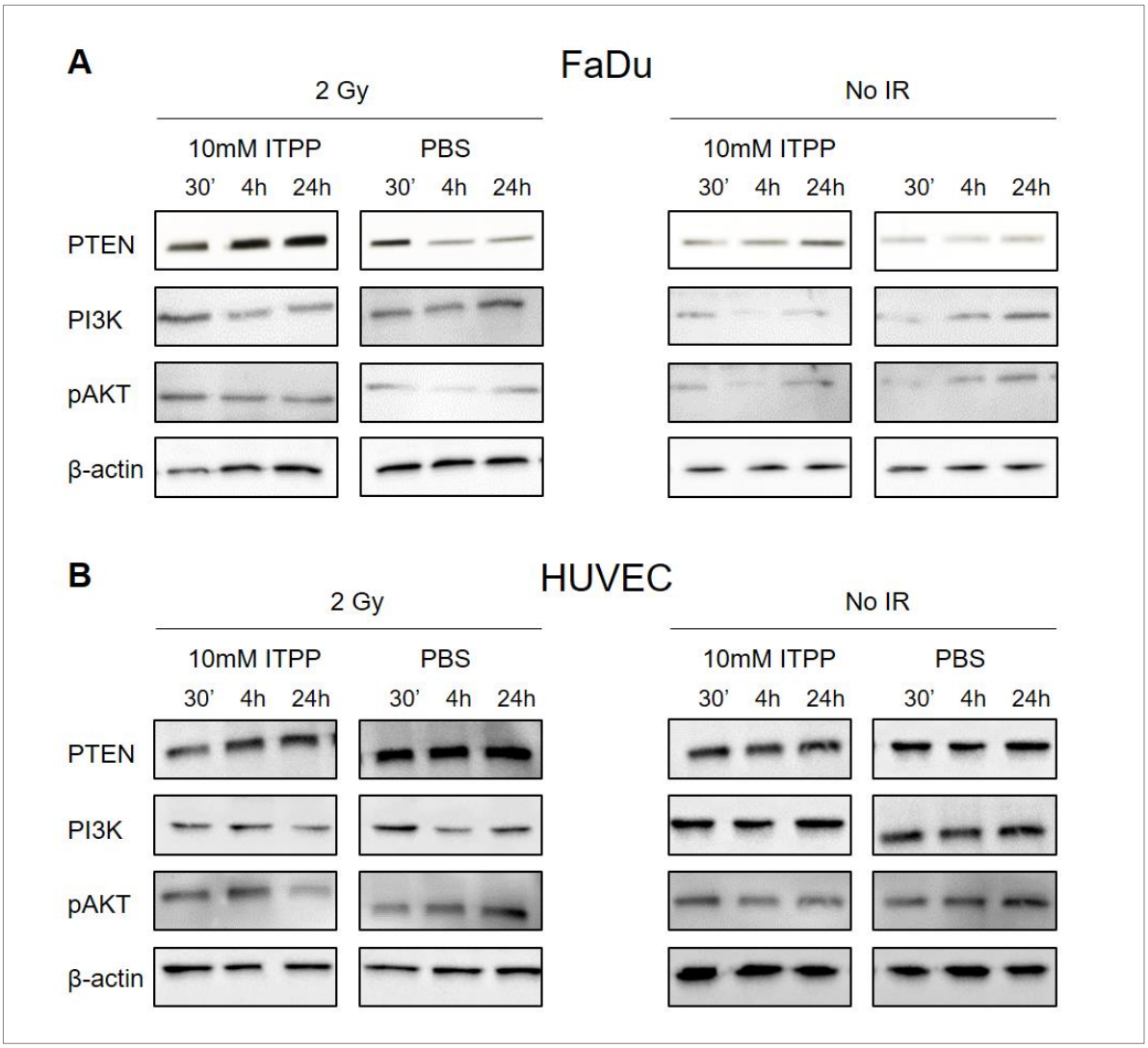


Figure 16 Representative Western Blots probed for different proteins within the PI3K/AKT pathway derived from A549 and FaDu whole cell lysates. The cells were pre-incubated for 2h with 10mM ITPP or PBS and harvested at different timepoints after irradiation with 2 Gy. **A)** An ITPP-dependent increase in PTEN level was demonstrated in FaDu cell line. In combination with irradiation, the increase in PTEN is more intense as compared to ITPP treatment only. pAKT levels were demonstrated to decrease concomitantly. **B)** The ITPP-dependent decrease in pAKT levels was also demonstrated in HUVECs. The decrease in pAKT was present in cells with ITPP monotreatment and in combination with irradiation.

3.2.3 Proliferation and clonogenic cell survival assays of FaDu cells demonstrated no difference upon ITPP treatment

Since the PI3K/AKT pathway has a major effect on proliferation and survival (1.7), ITPP treatment might have an impact on the proliferative activity of different cell lines. The proliferative activity of FaDu cells was assessed in 96-well plates with the colorimetric AlamarBlue assay (Biosource International). In order to investigate the impact of ITPP, the cells were incubated with 10mM ITPP or PBS for different time periods (2h, 6h and 24h) and irradiated with increasing doses (0, 5, 10 Gy). In comparison to FaDu cells that only received radiotherapy, ITPP treatment did not evoke any difference in proliferative activity alone or in combination with irradiation.

Similar results were obtained for ITPP treatment in combination with irradiation for the proliferative activity of A549 cells or HUVECs (data not shown).

To quantify the effect of ITPP activity on cellular survival and radiosensitivity, clonogenicity was determined. The clonogenic cell survival of FaDu cells pre-treated with 10mM ITPP or PBS for different time periods (2h, 6h, 24h) and irradiated with increasing dose of irradiation (0, 2, 4 and 6 Gy) did not show any difference regarding clonogenicity of the FaDu cells. The formation of colonies was allowed for 15 days (data not shown).

Collectively, these *in vitro* results demonstrated an ITPP-dependent increase of PTEN in hypoxic and normoxic conditions and a concomitant decrease in pAKT. Upon irradiation in combination with ITPP, the irradiation-induced increase in pAKT was abolished. Of note is, that ITPP in combination with irradiation did not promote any difference in proliferative activity of the tested cell lines (A549, FaDu, HUVECs), nor in clonogenicity in FaDu cells.

4 Discussion

The focus of this Master's Thesis was to address how a specific chemical compound, namely *Myo*-Inositoltrispyrophosphate, renders tumors more radiation-sensitive via modulation of the tumor hypoxia status.

Hypoxia can arise through several factors within a tumor leading to a selection of tumor cells with a more aggressive and treatment-resistant phenotype. Owing to hypoxia, not only chemotherapy is not properly delivered, even treatment outcome after surgery is worse [13, 15, 30]. The strongest impact of hypoxia can be observed when tumors are treated with radiotherapy;

Ionizing radiation leads to the production of ROS, which can induce DNA DSBs. Because of DNA DSBs, cells cannot properly divide and will experience mitotic catastrophe. A program will be initiated to prevent the formation of cells with incomplete chromosome numbers, leading to cell death or senescence [52]. The fixation of DNA DSBs requires oxygen. In normoxic cells, this fixation is possible, but not in hypoxic cancer cells. Therefore, in tissues with $pO_2 < 5$ mmHg (1%), the response to irradiation is up 2.5 to 3 times lower as compared to a well oxygenated (cancer) tissue [4, 19, 29]. For this reason, addressing tumor hypoxia and its modulation through different strategies is of great importance. To rise the pO_2 within the tumor, several possibilities are present:

To increase the delivery of oxygen, antiangiogenic treatment (in order to induce normalization of the tumor vasculature), the breathing of hyperbaric oxygen or other gases with high oxygen concentration have been preclinically and clinically tested. Another solution can be to reduce cellular oxygen consumption (e.g. by metformin) or to selectively eradicate hypoxic cells using hypoxia-activated prodrugs (e.g. by evofosfamide) (1.4).

In this Master's Thesis, a further approach to decrease hypoxic areas within tumors was explored: the use of *Myo*-Inositoltrispyrophosphate (ITPP). The aims of the thesis included validation of ITPP as an oxygen-modulator in an additional tumor model, and assessment of its capability to act as a radiosensitizer through its pO_2 increasing potential.

In the main part of this thesis, the mechanism underlying the tumor growth control upon ITPP treatment in combination with irradiation was investigated. It was demonstrated that upon ITPP treatment, more DNA DSBs could be induced and stabilized ("fixed") in initially hypoxic cells, compared to hypoxic cells within tumors which only received radiotherapy. An additional side project included the *in vitro* investigation on the PI3K/AKT pathway, since ITPP structurally resembles the two molecules PIP_2 and PIP_3 which act upstream of this pathway. The PI3K/AKT pathway is relevant for cancer research, as it is one of the major mediators of cell growth and survival, and is often hyperactivated in cancer cells.

4.1 ITPP as an oxygen-modulator sensitizes hypoxic tumors for radiotherapy

Previously it was demonstrated, that ITPP treatment can modulate the oxygen-content within A549-derived tumor xenografts. Already 2h after ITPP injection, a significant decrease in a hypoxia-detecting bioluminescent-based signal was measured, which indicates an increase in oxygen availability within the tumor tissue (Supplementary 1). This reporter protein consists of an oxygen dependent degradation domain fused to luciferase and is expressed in the respective cell line of interest.

Different tumor entities vary in their susceptibility to develop hypoxia due to several factors: the genetic make-up and evolution of the tumor cell, and the size and location within the body. A high heterogeneity of the oxygen status also exists within the same tumor entities. In order to probe ITPP in an additional tumor entity, the oxygen-modulating capacities of ITPP were also assessed within tumor xenografts derived from the head and neck cancer cell line FaDu, in which hypoxia is less prevalent compared to A549-derived xenografts [47].

In FaDu-derived xenografts, ITPP treatment lead to a significant increase in intratumoral oxygen 2h after the second ITPP treatment, which was applied on two consecutive days (Figure 9B). These results present ITPP as a competent oxygen-modulator for xenografts derived from two different cell lines. The characteristics of ITPP as a radiosensitizer were verified by an efficacy study in A549-derived xenografts (Figure 10B) and previously in FaDu-derived xenografts (Figure 10A and [47]) as well. For radiotherapy in combination with ITPP, there is a significant survival prolongation in comparison to control irradiated mice caring xenografts. These results were expected because of the *oxygen-enhancing effect*: Through the increase in pO_2, more ROS are generated, leading to an increased stabilization ("fixation") of DNA DSBs in initially hypoxic zones of the tumors (or at least partially). This eventually translates into more effective tumor cell killing.

This effect underlies the right shift of the oxygen-dissociation curve of hemoglobin upon ITPP treatment (Figure 6 and Figure 17) [53]. Because of an abnormal vasculature or an altered metabolism, cancer cells are not accurately delivered with oxygen. The right-shift within the oxygen-dissociation curve upon ITPP treatment resembles the facilitated liberation of oxygen from hemoglobin. Cancer cells with low oxygen content will receive more O_2 and DNA damage can be fixed.

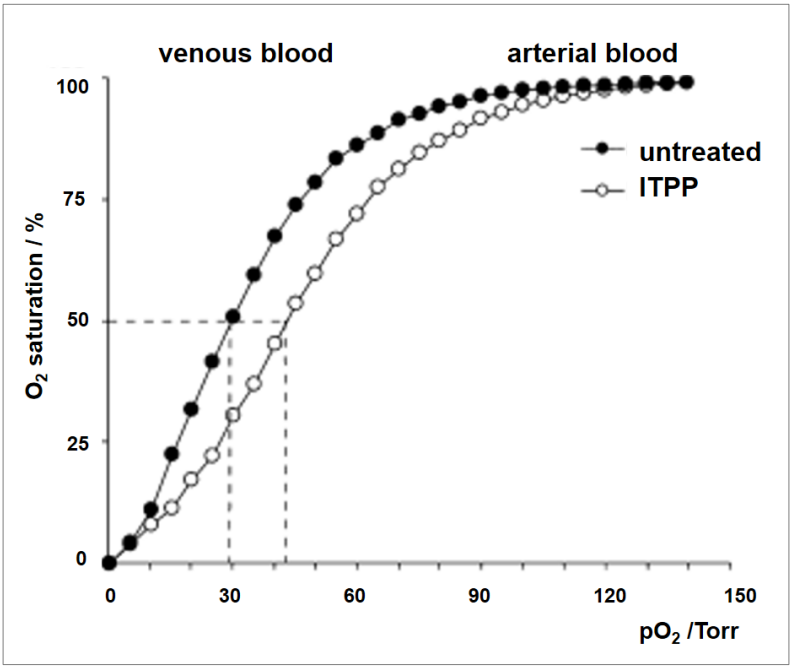


Figure 17 Shift of the oxygen-dissociation curve of hemoglobin to the right upon ITPP treatment, indicating the facilitated liberation of oxygen from hemoglobin. Figure adapted from [61].

Since radiotherapy is one of the most relevant treatment modalities, alone or in combination with surgery, chemotherapy or molecularly targeted therapy, it is very important to invest in the modulation of oxygen within a tumor in order to increase treatment outcome and patient survival. Of interest is, that ITPP is currently being tested in a phase II clinical trial in combination with FOLFOX chemotherapy for non-resectable visceral tumors in the Department of Visceral Surgery at the University Hospital of Zurich [54].

4.2 Hypoxic tumor cells experience more DNA DSBs upon irradiation in combination with ITPP

Ionizing radiation primarily kills tumor cells by the induction of un- or falsely repaired DNA DSBs. Mechanistic investigations underlying the tumor growth delay, or the survival advantage respectively, upon irradiation in combination with ITPP were performed. While tumor control in response to extended irradiation protocols is due to full eradication of all proliferating cells (cell death or treatment-induced senescence), tumor growth delay (as an endpoint in preclinical *in vivo* experiments with irradiation) is most probably due to only partial killing of the full number of cells present in a tumor xenograft and regaining of tumor proliferative activity of the surviving tumor cells.

To get deeper insight of the underlying mechanism of the tumor growth delay, tumor xenografts derived from the FaDu cell line were irradiated and the amount of DNA DSBs were quantified using the DNA

DSBs marker γH2AX. A short term (0.5h after irradiation) and a long term (24h after irradiation) timepoint were used to assess initial and residual DNA DSBs within hypoxic and normoxic regions. To stratify between zones with low pO_2 (below 10 mmHg) and sufficient oxygen prior to treatment, marking of hypoxic zones by pimonidazole was used. For each tumor sample, three zones in hypoxic and three zones in normoxic areas of the tumor were chosen. For each zone, the γH2AX foci were counted in at least 100 cells. Mitotic, necrotic or cells with micronuclei were excluded from the analysis. In average, the same number of cells within a defined zone had to be excluded, proposing that this does not influence the result in average counted γH2AX foci per cell.

Within normoxic zones, there is no difference in the number of γH2AX foci per cell for ITPP treatment alone. This indicates that ITPP does not have an additional effect regarding DNA DSBs induction in normoxic cells (Figure 13A).

Hypoxic cells within unirradiated xenografts share a similar distribution of γH2AX foci per cell with normoxic cells and display no significant difference in average γH2AX foci per cell (Figure 13B).

Regarding the number of foci within hypoxic cells shortly after irradiation (short term timepoint 0.5h after irradiation), there are significantly more γH2AX foci per cell in initially hypoxic cells which were ITPP treated right before irradiation (Figure 14A). This strongly supports the *oxygen-enhancing effect*, indicating that the amount of ROS and subsequent induction and fixation of DNA DSBs is lower in hypoxic cells without ITPP treatment. This is also clearly demonstrated in the number of cells with no γH2AX foci: Upon ITPP treatment, almost no pimonidazole-positive (i.e. hypoxic prior to ITPP-treatment) cell remained undamaged regarding DNA DSBs (Figure 14A right), whereas for control irradiated pimonidazole-positive cells, almost 10% of cells did not respond, meaning they have no radiation-induced DNA DSBs. Cells which experience almost no irradiation-induced DNA DSBs are said to not respond to radiotherapy.

For the long term timepoint after irradiation (24h), the average number of γH2AX foci per cell decreases for either treatment, indicating that DNA DSB repair was active.

The remaining foci represent residual DNA damage that will eventually lead to eradication or senescence of the affected cells (Figure 14C and 14D). Again, significantly more DNA DSBs could be detected in ITPP treated, irradiated pimonidazole-positive tumor xenograft zones in comparison to PBS-treated, irradiated pimonidazole-positive tumor xenograft zones (Figure 14C). As such, this quantitative difference in the amount of residual DNA DSB could translate into a more efficient treatment outcome. Of note is, that the residual γH2AX foci per cell is dependent on the initially induced DNA DSBs.

The reason for the surprising difference in residual foci within normoxic cells for ITPP and control treated and irradiated cells (Figure 14D) could be that some zones were counted as normoxic when they

were actually slightly hypoxic but still less radiation-responsive. Pimonidazole staining is only accurate for a pO_2 level below 10mmHg. In such case, pimonidazole-staining does not identify such slightly-hypoxic cells. Furthermore, DNA-repair capacity could also be different in these intermediate zones (4.3).

4.3 ITPP acts on the PI3K/AKT pathway

The PI3K/AKT pathway is involved in a variety of cellular functions including growth, proliferation, metabolism, angiogenesis and survival. Hyperactivation of the PI3K/AKT pathway is linked to tumor development, progression and resistance to cancer therapies, such as radiotherapy. Furthermore, pAKT is involved in a correct DNA damage response (DDR). The *in vitro* ITPP treatment in combination with irradiation demonstrated an ITPP-dependent increase in PTEN levels, which is the main antagonist of this pathway. Concomitantly, a decrease in pAKT levels was observed.

PI3K activity could be inhibited through binding of ITPP within its catalytic cleft, which is usually occupied by PIP_2. The reduced phosphorylation of PIP_2 leads to decreasing levels of PIP_3. PIP_3 is necessary to recruit AKT to the plasma membrane where its activation through phosphorylation will be mediated through other kinases. Through reduced levels of PIP_3, pAKT levels decrease simultaneously – leading beside other effects –to a dysregulated DDR. Thus, the residual DNA damage could be due to reduced DNA DSB repair in irradiated and ITPP treated cells, and enhanced DNA DSB repair in control irradiated hypoxic cells (3.1.3). However, such quantitative correlations need to be demonstrated also as part of *in vitro* experiments, which were not performed in this thesis. Furthermore, the mechanism of ITPP-dependent increase of PTEN levels in different target cells was not investigated. Notably, it was not possible to demonstrate reduced clonogenic survival of FaDu cells pretreated with ITPP prior to irradiation. These results argue for a minor role of ITPP on the PI3K/AKT pathway in respect to act as a radiosensitizer *in vitro*.

5 Conclusion

The aims of this Master's Thesis included probing of the capacity of ITPP as a reoxygenator and radiosensitizer in tumor xenografts derived from different cancer cell lines (A549 and FaDu). Further, the mechanism underlying the tumor growth delay upon irradiation in combination with ITPP was investigated.

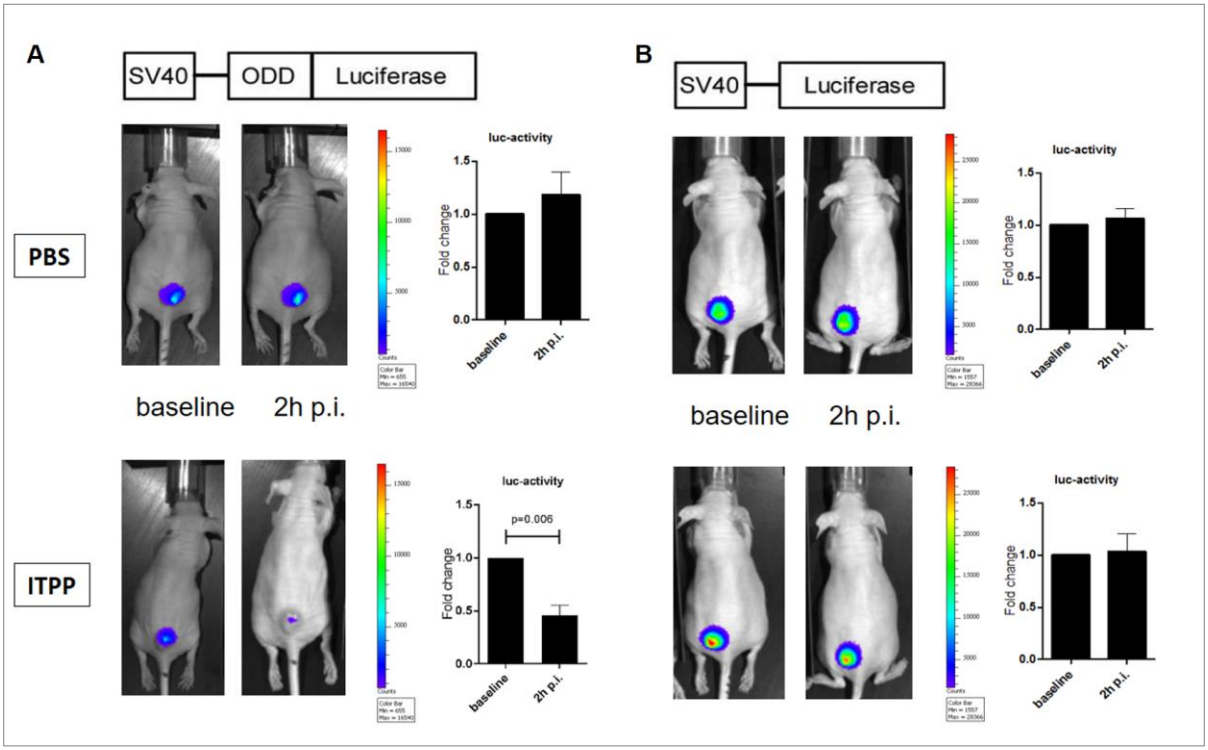
It was demonstrated that ITPP leads to reoxygenation of hypoxic areas within tumors. These areas could then be linked to enhanced irradiation-induced initial and residual DNA DSBs in comparison to untreated and only irradiated hypoxic tumor areas. These increased levels of DNA DSBs are most probably responsible for the improved treatment response in tumor xenografts upon treatment with ITPP in combination with irradiation as compared to irradiation alone. Furthermore, it was also demonstrated that ITPP interferes with the PI3K/AKT signal transduction cascade. However, these intriguing results could not be further extended due to time reasons and must be examined in further detail in the future. Right now, several additional preclinical studies are ongoing *in vivo* in the Laboratory of Applied Radiobiology at the University Hospital of Zurich (USZ), to address the impact of ITPP in combination with irradiation in more advanced tumor models including orthotopic tumor models in immunocompetent mice.

Overall, ITPP leads to a significantly better radiotherapy outcome *in vivo*. Therefore, it should be considered as a neoadjuvant treatment for radiotherapy. Importantly, as ITPP demonstrates its greatest power in reoxygenating hypoxic tissue, it should be administered to patients with a "hypoxia-driver phenotype". In cancer treatment, the biggest chance for successful treatment lays in targeting the pathophysiological peculiarities of a certain cancer, and hypoxia is definitely one of those. Patients with the "hypoxia-driver phenotype" belong to a subgroup of cancer patients that will benefit most from the capabilities of ITPP to increase oxygen delivery into hypoxic tissue.

Supplementary

All the preliminary data in this thesis and the rationale to combine ITPP with radiotherapy is based on the results of Ivo Grgic, PhD student at the USZ and tutor of this work. Prior to investigating the anti-tumor effect of ITPP in combination with radiotherapy, an optimal dose regimen of ITPP and the ideal time-window for irradiation were defined in order to maximize the biological effect upon treatment. This was achieved by using the hypoxia-specific bioluminescent imaging approach described in this thesis (2.3.4) [49]. The reoxygenation capability of ITPP was assessed in xenografts derived from the non-small cell lung carcinoma (NSCLC) cell line A549 (Supplementary 1), since this cell line was already validated in view of hypoxia in several other projects within the Laboratory of Applied Radiobiology at the USZ. Dose escalation studies revealed a maximal tolerated dose of 3g/kg bodyweight ITPP, intraperitoneally injected on two consecutive days. The bioluminescence signal was always measured 2h after treatment injection in addition to a baseline signal.

The reoxygenation of ITPP is already significant 2h after the first ITPP injection (Supplementary 1A).



Supplementary 1 Representative images showing the effect of PBS and ITPP on tumor reoxygenation as determined by a **A)** hypoxia-specific and a **B)** hypoxia-unspecific bioluminescent *in vivo* imaging in A549-derived xenografts. Imaging was performed immediately before and 2h after PBS/ITPP (3mg/kg bodyweight) injection. There is a significant decrease in bioluminescent signal, indicating an increase in oxygen, 2h after ITPP treatment.

Previous *in vivo* efficacy-oriented tumor growth delay studies were carried out in xenografts derived from the head and neck squamous cell carcinoma (HNSCC) cell line FaDu (and not in A549-derived tumor xenografts as in investigated in this thesis), because of the interest claimed by NormOxys and the clinical relevance of HNSCC. Xenografts were treated on two consecutive days (ITPP/PBS) and after the second injection, tumor xenografts were irradiated with 10 Gy. Whereas ITPP treatment did not show any difference compared to control treatment (PBS), treatment with ITPP prior to irradiation significantly increased the tumor growth delay in comparison to irradiation alone (Figure 10A).

In the scope of this work, complementary bioimaging and efficacy-oriented experiments were performed in FaDu-derived xenografts and A549-derived xenografts respectively. DNA-damage related endpoints were assessed in tumor xenografts derived from FaDu cells. Additionally, PI3K/AKT pathway-related experiments were performed in several cell lines in *vitro.*

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